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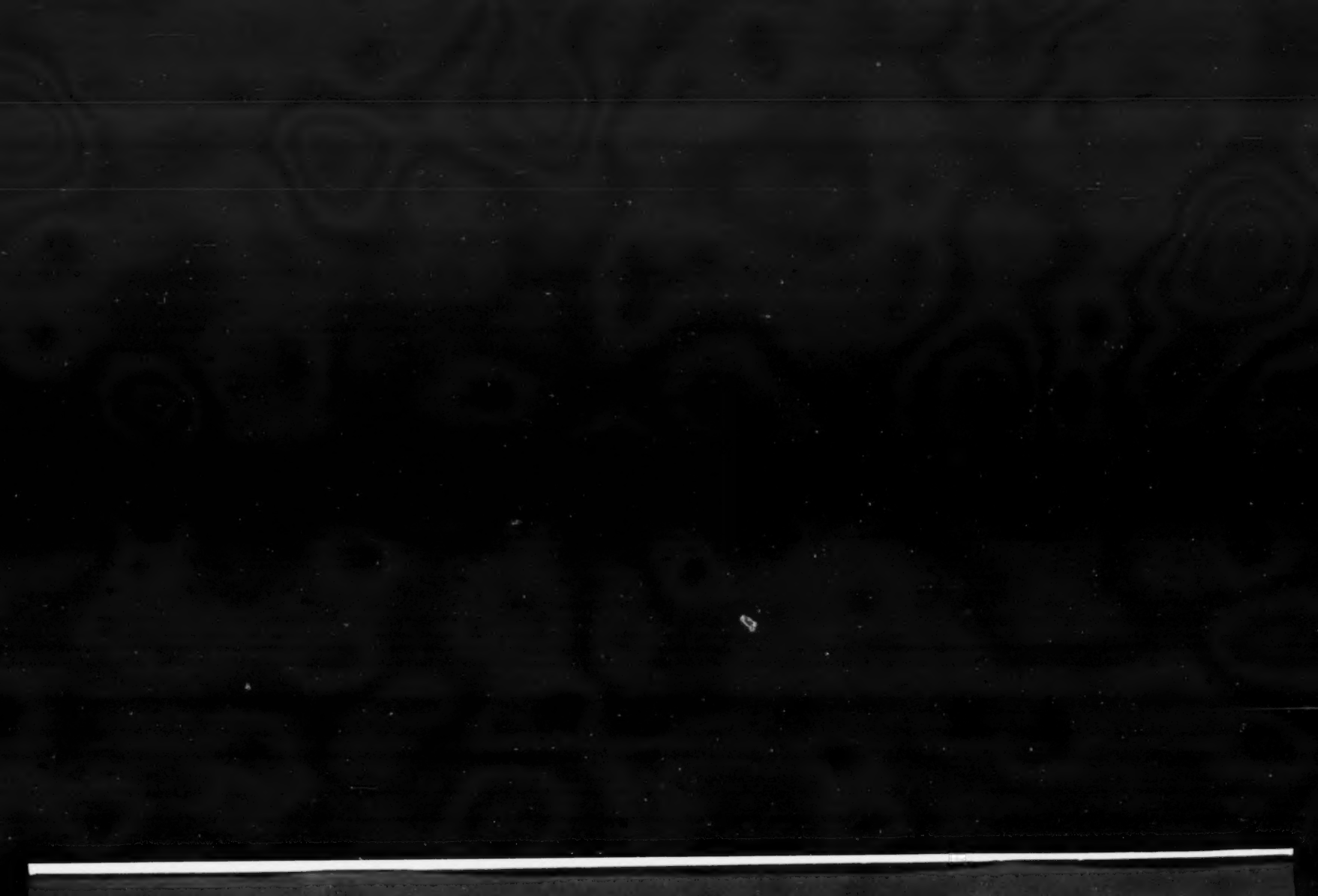

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VOL. 29 APRIL, 1950 NUMBER 2

# EFFECTS OF COLD, ASCORBIC ACID, AND AGE ON "FORMALDEHYDE-INDUCED" ARTHRITIS IN THE WHITE RAT[1]

BY LOUIS-PAUL DUGAL[2]

## Abstract

Ascorbic acid has been claimed to have a definite antirheumatic activity in humans. It has also been found to have a slight protective action against experimental arthritis in animals. We have been able to show that ascorbic acid has a definite and significant beneficial effect on the same disease in adult rats, but not in young animals. Moreover, our results show that the formaldehyde-induced arthritis is greatly aggravated by exposure to cold.

The rheumatic and the so-called "collagen" diseases have been referred to by Selye as being diseases of adaptation (13, 14). According to his theory, the otherwise beneficial and useful general-adaptation syndrome "may derail under abnormal conditions, perhaps because the production of gluco-corticoids cannot keep in pace with the excessive elaboration of mineralo-corticoids, and become itself the principal cause of disease" (15). In other words, a successful answer to stress should imply an increased gluco-corticoid hormone production, as compared to the one of the mineralo-corticoids, type D.C.A.

In applying that assumption to the study of formaldehyde-induced arthritis, we felt that long exposure to cold (contrarily to short exposures) (15) should probably aggravate the disease, on the ground that under such circumstances, cold seems to be a continuous stress (19) which calls for the continuous need of the adrenals even in adapted animals, contrarily to what happens in chronic muscular exercise (3). Besides, we knew that resistance to long exposures to cold is decreased by sodium chloride injections (6), a fact which is to be compared to the one that sodium chloride sensitizes animals to mineralo-corticoids (such as D.C.A.) (13, 14). For analogous reasons, and also on account of what exists really in nature, it seemed to us that the age factor should be studied.

Finally, owing to the fact that ascorbic acid increases resistance especially in cases where the exhaustion of the adrenal function is likely to happen

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*Contribution from the Département de recherches en Acclimatation, Faculté de Médecine, Université Laval, Québec, P.Q., with financial assistance from the Defence Research Board of Canada.*

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(4, 5, 6, 8, 11, 16, 18, 21), we were naturally interested to know whether that substance would have a cortisone stimulating action (ACTH-like) on the experimental arthritis elicited by formaldehyde. In that connection, the recent results of Schaffenburg, Masson, and Corcoran (12) are of great interest; those authors have found that "cortisone inhibits many of the effects of scurvy in the guinea-pig while desoxycorticosterone aggravates the condition".

Ascorbic acid in combination with D.C.A. has been studied very often in recent years for its effects on rheumatic changes in man and animals, but the results obtained are conflicting.

Ascorbic acid alone, in very large doses (4 gm. per day) has been claimed to have an "antirheumatic activity" in man (10); a slight protection by the same substance against experimental arthritis in rats has been described by Brownlee (1) who used 20 mgm. a day: the small beneficial effect was obtained only in normal young (100-110 gm.) rats and not in adrenalectomized ones, a significant finding to us. More recently, however, Coutu and Selye (2) have failed to confirm the statistical significance of Brownlee's results.

Our aim has been to try the effects of large doses of ascorbic acid* on young and adult rats for its possible protection against formaldehyde-induced arthritis.

## Experimental Procedure

(1). The animals were injected with 0.1 cc. of 2% formaldehyde in the left hind paw, just beneath the plantar aponeurosis, and the right hind paw of the same animal always served as a control.

(2). The frequency of the formaldehyde injections varied with the different experiments and is indicated for each of them.

(3). Changes in the inflammatory processes have been estimated by measuring the volume of both the injected leg and the noninjected one by displacement of water. Both legs were immersed in turn, up to the knee joint, in a beaker of water which was placed on a torsion balance; the difference (obtained by weight) between the volumes of the two legs corresponded to the swelling (or to the increase in swelling in the cases of exposures to cold) due to the injection of formaldehyde. During measurements, the animals were kept under ether anesthesia and placed right over the beaker on a board provided with an opening allowing for the immersion of one hind leg at a time; measurements of the inflammatory changes were always made without the operator knowing to which group the animals belonged.**

(4). The temperature in the cold room was maintained at 1° C. ± 1° C.

(5). Three main experiments were made using male white rats of 320 gm. in average weight in the first two, and of 140 gm. in the last one. The first two experiments were designed to estimate the effects of cold and large doses

* *"Redoxon forte" from Hoffman-Laroche, Montreal.*

** *That method appeared to be very faithful: repeated measurements of the right hind paw (not injected) of the same animals kept at room temperature gave reproducible results within 1% of the average value.*

of ascorbic acid on experimental arthritis in adult rats. In each of them, the animals were divided into two groups, one receiving sodium ascorbate (by mouth, with a tuberculin syringe) and one being kept as a control group (untreated); both groups were subdivided, half of each being exposed to cold, the other half being kept at room temperature. The same procedure has been followed in the third and last experiment which was performed on young rats.

TABLE I

SWELLING OF INJECTED LEG (IN CC.)

| Figure | Days | Ascorbic acid group | Control group | Value of "t"* |
|---|---|---|---|---|
| 1 | 2 | 0.32 ± .060** | 0.625 ± .076 | 3.23 |
| " | 6 | 0.30 ± .050 | 0.69 ± .117 | 3.07 |
| " | 8 | 0.307 ± .059 | 0.566 ± .085 | 2.51 |
| " | 11 | 0.515 ± .057 | 0.637 ± .069 | 1.37 |
| " | 14 | 0.52 ± .053 | 0.59 ± .067 | 0.82 |
| 3 | 1 | 0.31 ± .036 | 0.47 ± .039 | 3.02 |
| " | 12 | 0.297 ± .043 | 0.424 ± .045 | 2.05 |
| " | 14 | 0.418 ± .034 | 0.555 ± .044 | 2.49 |
| " | 21 | 0.27 ± .059 | 0.33 ± .045 | 0.81 |
| 5 | 4 | 0.254 ± .032 | 0.315 ± .032 | 1.36 |
| " | 10 | 0.276 ± .033 | 0.332 ± .027 | 1.33 |
| " | 14 | 0.236 ± .040 | 0.207 ± .032 | 0.57 |
| " | 37 | 0.55 ± .082 | 0.63 ± .092 | 0.65 |
| " | 45 | 0.54 ± .075 | 0.62 ± .072 | 0.77 |

NOTE: *Numerical data of Figs. 1, 3, and 5.*

* *"t" values were calculated according to the formula:* $t = \frac{Difference}{\sqrt{\left(\frac{\sigma x_1}{\sqrt{N_1 - 2}}\right)^2 + \left(\frac{\sigma x_2}{\sqrt{N_2 - 2}}\right)^2}}$

** *Standard error.*

## Results

(1). In the first experiment, which was only a preliminary one, 18 rats were used. Fig. 1 shows the effect of ascorbic acid on formaldehyde-induced arthritis, the results obtained in the cold room and at room temperature having been added. Such a way of presenting the results might mask possible differences as to the effect of ascorbic acid at different temperatures, but that specific point will be taken care of and clarified later.

In Fig. 1 (as well as in the following figures), the ordinate represents the differences in volume (cc. × 100) between the injected leg and the noninjected one. In other words, it gives the measure of the *swelling due to the injection of formaldehyde.* It may be seen that two days after the first injection, the increase in volume in the control group was a little more than 0.60 cc. whereas the ascorbic acid (25 mgm. daily) treated group showed a swelling of only 0.30 cc., a difference of 95% which is highly statistically significant ($t = 3.23$). A second injection of formaldehyde was given three days later, and the new measurements made the day after showed a still larger difference, highly

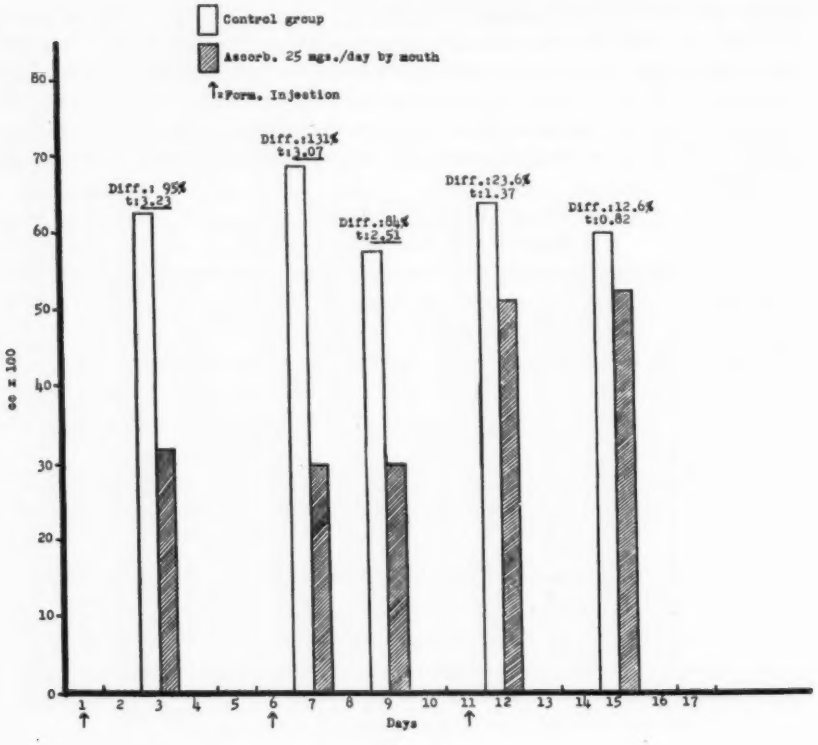


FIG. 1.

## TABLE II

SWELLING OF INJECTED LEG (IN CC.)

| Figure | Days | Cold room | Room temperature | Value of "t" |
|---|---|---|---|---|
| 2 | 2 | 0.58 ± .10 | 0.415 ± .07 | 1.35 |
| " | 6 | 0.62 ± .128 | 0.40 ± .096 | 1.38 |
| " | 8 | 0.564 ± .115 | 0.36 ± .055 | 1.56 |
| " | 11 | 0.664 ± .082 | 0.52 ± .048 | 1.50 |
| " | 14 | 0.713 ± .057 | 0.438 ± .024 | 4.50 |
| 4 | 1 | 0.42 ± .051 | 0.37 ± .028 | 0.86 |
| " | 12 | 0.53 ± .043 | 0.24 ± .030 | 5.58 |
| " | 14 | 0.57 ± .039 | 0.42 ± .038 | 2.78 |
| " | 21 | 0.51 ± .040 | 0.138 ± .025 | 7.91 |
| 6 | 4 | 0.324 ± .026 | 0.247 ± .024 | 2.20 |
| " | 10 | 0.41 ± .025 | 0.22 ± .018 | 6.33 |
| " | 14 | 0.33 ± .035 | 0.10 ± .020 | 5.75 |
| " | 37 | 0.80 ± .052 | 0.22 ± .023 | 10.18 |

NOTE: *Numerical data of Figs. 2, 4, and 6.*

statistically significant. A third measurement made two days later showed the same phenomenon. It must be pointed out, however, that here the protective action of ascorbic acid was no longer demonstrable after the third injection of formaldehyde.

Fig. 2 shows that cold aggravates the swelling due to formaldehyde, though the results are not statistically significant except for the last measurement

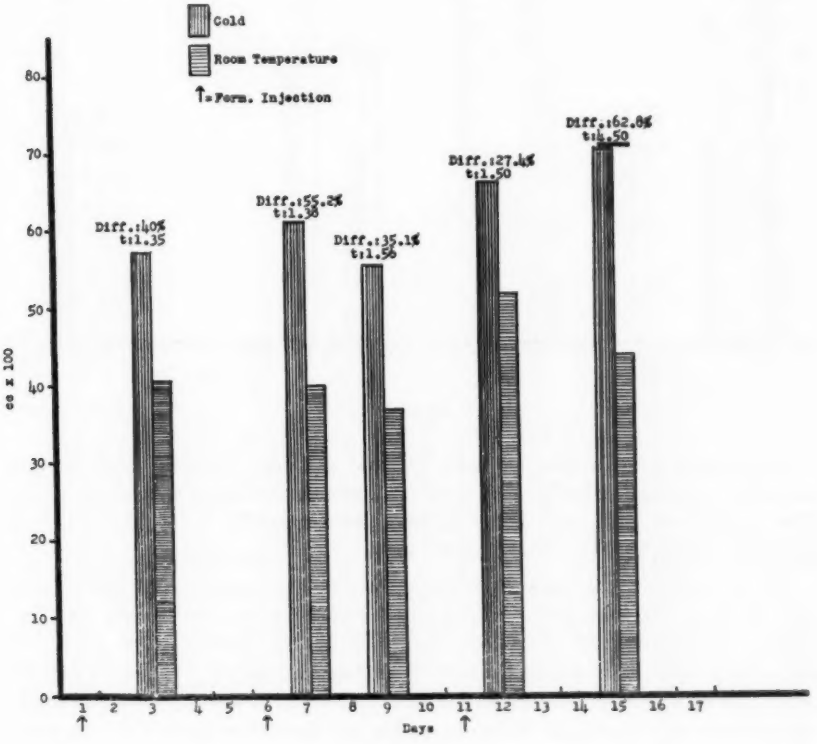


FIG. 2.

which was made after three injections of formaldehyde and an exposure to cold of over one month, and which shows an increased volume of 63%, statistically highly significant ($t = 4.50$). (Compare with Figs. 4 and 6.)

(2). The second experiment was a repetition of the first one on a larger group of animals (a total of 52).

Fig. 3 shows the same protective effect for ascorbic acid as Fig. 1 did. The treated animals, here, received 200 mgm. daily of ascorbic acid, under the form of ascorbate.

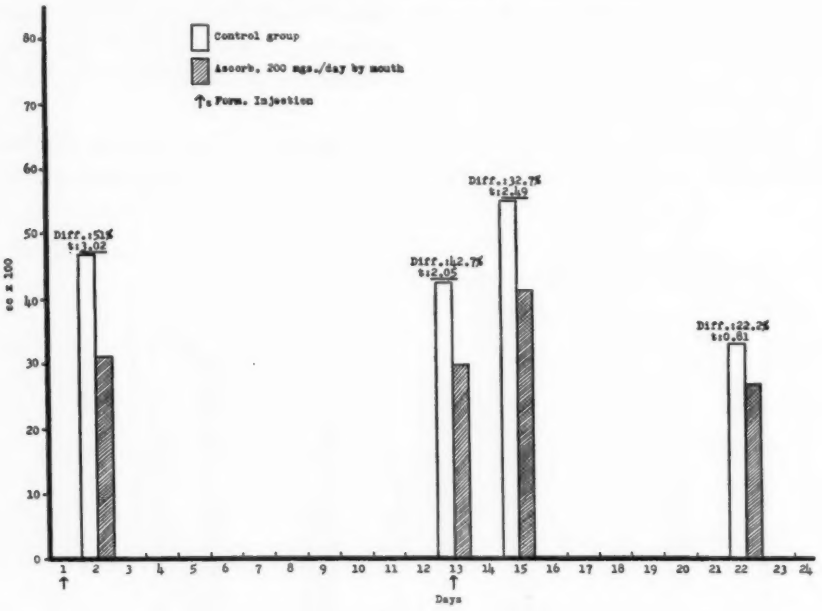


FIG. 3.

Fig. 4 shows again that exposure to cold (22 days in all) aggravates the so-called formalin-arthritis, this time in a more convincing way than in the first experiment. Except for the first measurement the differences are striking and highly statistically significant.

(3). The third experiment was made on young growing rats weighing 140 gm. on the average at the beginning of the experiment. Here, the protective action of ascorbic acid (150 mgm. daily by mouth) seems to be much less important (Fig. 5). Measurements have been made at different intervals after the injections of formaldehyde, but never has it been possible to make conspicuous any significant difference, although we had groups of 25 rats each.

The fact is that the actual volume of the injected leg is much smaller, in a highly significant statistical way, in the young animals than in the old ones, as can be seen from Table III, although both groups were exposed at the same time to the same cold temperature and have about the same volumes for the noninjected right leg (at the time the measurements were made, the body weight of the young rats had already attained a value of 180 gm.). The groups referred to in Table III are the control groups of the second and of the third experiments, the groups that did not receive any ascorbic acid. In other words, it seems that *the formaldehyde-induced arthritis* is much less severe in young than in adult rats, and that would explain why the protective action of ascorbic acid cannot be made conspicuous in the young animals.

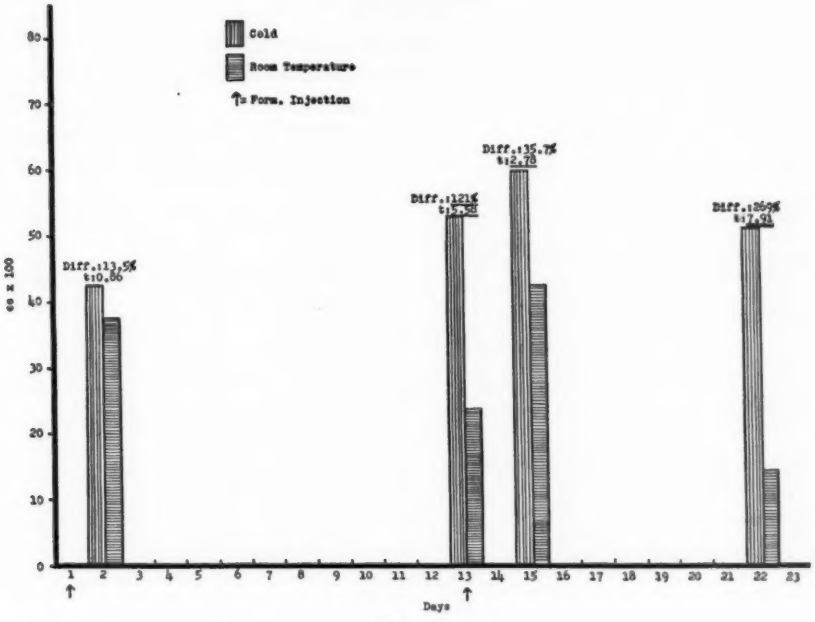
Cold
Room Temperature
↑= Form. Injection
Diff.:13.5%
t:10.86
Diff.:121%
t:5.58
Diff.:35.7%
t:2.78
Diff.:269%
t:7.91
oo x 100
Days

FIG. 4.

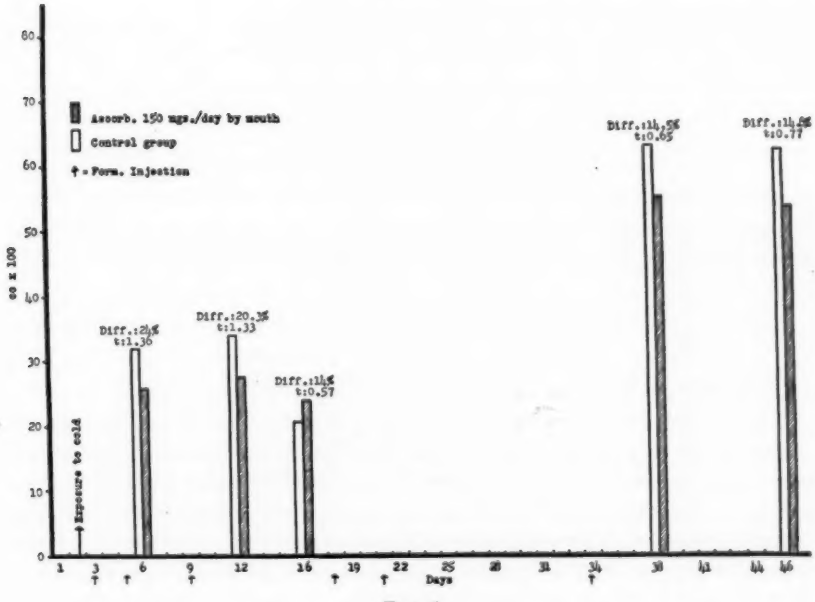
Ascorb. 150 mgs./day by mouth
Control group
↑ = Form. Injection
Diff.:2½%
t:1.36
Diff.:20.3%
t:1.33
Diff.:11½%
t:0.57
Diff.:114.5%
t:0.65
Diff.:114.5%
t:0.77
↑ Exposure to cold
oo x 100
Days

FIG. 5.

TABLE III

EFFECT OF AGE ON EXPERIMENTAL ARTHRITIS IN RAT

| Control groups | Left leg injected with formaldehyde, volume in cc. | Right leg not injected, volume in cc. |
|---|---|---|
| Rats of 320 gm., 1 injection, exposed to cold 2 days | 1.85 ± 0.068 | 1.27 ± 0.032 |
| Rats of 148 gm., 2 injections, exposed to cold 4 days | 1.59 ± 0.048 | 1.24 ± 0.037 |
| Difference | 16.3% | Nil |
| *t* | 3.13 | |
| Rats of 320 gm., 2 injections, exposed to cold 15 days | 1.89 ± 0.080 | 1.23 ± 0.068 |
| Rats of 148 gm., 2 injections, exposed to cold 4 days | 1.59 ± 0.048 | 1.24 ± 0.037 |
| Difference | 18.8% | Nil |
| *t* | 3.22 | |
| Rats of 320 gm., 2 injections, exposed to cold 15 days | 1.89 ± 0.080 | 1.23 ± 0.068 |
| Rats of 148 gm., 3 injections, exposed to cold 15 days | 1.51 ± 0.047 | 1.21 ± 0.033 |
| Difference | 20.5% | Nil |
| *t* | 4.13 | |

In the latter, it took six injections of formaldehyde before the swelling of the left paw (in comparison with the right one) could attain values comparable to the ones obtained after one or two injections in the older animals.

Contrarily to what has just been described for the effect of ascorbic acid in those young rats, the effect of cold (40 days in all) is very striking from the beginning to the end of the experiment. Differences as great as 260% and very highly significant ($t = 10$) have been obtained (Fig. 6).

So far, for clarity purposes, results have been presented by adding the ones obtained at room temperature and in the cold room, when the comparison was made between the group receiving ascorbic acid and the control one, and by adding the ones obtained by both groups at room temperature and in the cold room, when the effect of temperature was being investigated. Fig. 7 is another presentation of the second experiment (Figs. 3 and 4), and it shows that the results are not altered by either way of presenting them, so far as the

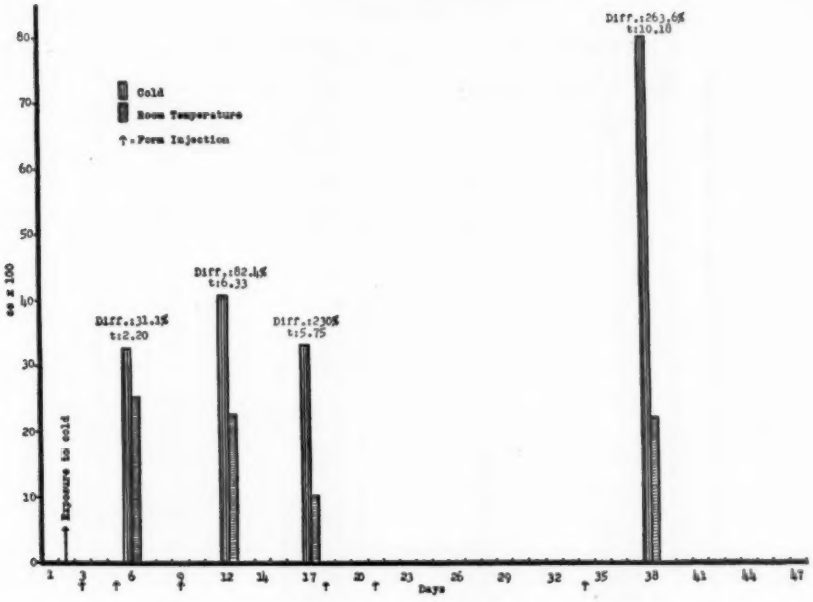


FIG. 6.

TABLE IV

SWELLING OF INJECTED LEG (IN CC.)

| Days | Cold room | | | Room temperature | | |
|---|---|---|---|---|---|---|
| | Ascorbic acid group | Control group | Value of "t" | Ascorbic acid group | Control group | Value of "t" |
| 1 | 0.24 ± 0.066 | 0.58 ± 0.045 | 4.30 | 0.37 ± 0.031 | 0.36 ± 0.051 | — |
| 12 | 0.44 ± 0.086 | 0.58 ± 0.047 | 1.42 | 0.20 ± 0.036 | 0.28 ± 0.054 | 1.25 |
| 14 | 0.465 ± 0.040 | 0.656 ± 0.055 | 2.80 | 0.386 ± 0.057 | 0.462 ± 0.054 | 0.97 |

NOTE: *Numerical data of Fig. 7.*

effects of ascorbic acid and cold are concerned. It shows something more: that the effect of ascorbic acid is more beneficial in the cold room; at room temperature, the protective action of ascorbic acid is slight and never significant. It must be added that there is only one instance in which the increase in volume has been smaller in the cold room than at room temperature: that happened for the ascorbic acid group 24 hr. after the first injection of formaldehyde which coincided with the entrance into the cold room. The difference has a value of 35% and was not statistically significant ($t = 1.78$).

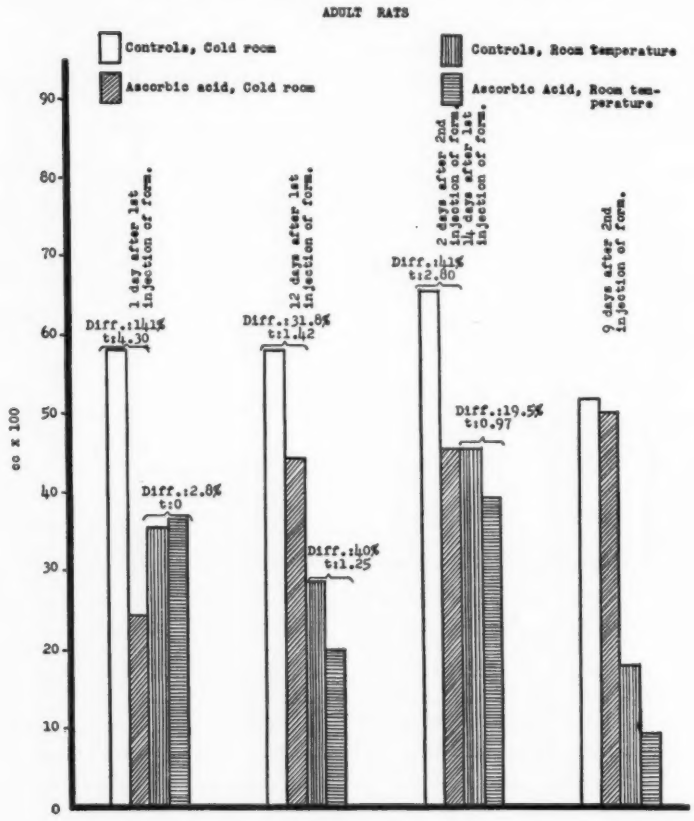


FIG. 7.

In young rats (Fig. 8, based on the same results as Figs. 5 and 6), there is no significant effect of ascorbic acid on the process, neither in the cold room nor at room temperature. There again, the effect of cold is clear.

(4). *Other observations.*—In rats exposed to cold and injected with formaldehyde, oedema of the penis appears much sooner and is far more intense than the one normally encountered after long exposures to cold alone (9); that effect is strikingly diminished by large doses of ascorbic acid, both in frequency and intensity. After 31 days of exposure to cold (+ 1° C.), during which period the rats received six injections of formaldehyde (0.1 cc. of a 2% solution, the last one being given on the 30th day) all control animals (e.g. 100%) (receiving no extra ascorbic acid) showed some oedema of the penis, exceedingly intense for most of them, while 60% of the group receiving 150 mgm. per day of ascorbic acid did not show the slightest trace of oedema and the remaining 40% showed only a light manifestation of it. Forty days later,

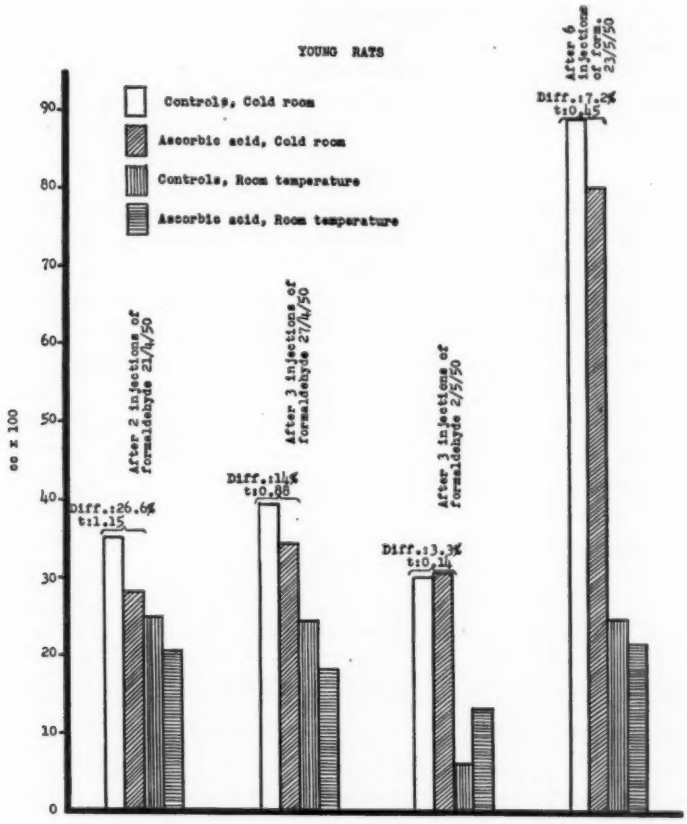


FIG. 8.

in other words, 41 days after the last injection of formaldehyde and after the exposure to cold had already lasted 71 days, none of the rats receiving the extra vitamin showed any sign of oedema, while 60% of the control animals still showed it. It seems that the swelling of the penis was due to the combined effect of formaldehyde and cold; in fact, long after the last injection of formaldehyde in the cold room, that pathological condition subsided partially in control animals and totally in ascorbate-treated rats, even if the animals were kept *constantly* in the cold room; on the other hand, the injection of formaldehyde produces no such oedema at room temperature. In the experimental conditions used (temp. + 1° C., rats weighing around 150 gm. when they were first exposed to cold), there was another explosion of the same disease *after six months* of exposure to cold, this time really due to the chronic exposure to the relatively low temperature (8), no more injection of formaldehyde having been given after the 30th day of exposure to cold. This time

again, there was a difference in the frequency and severity of the oedema of the penis between the two groups: all remaining animals of the control group (10 out of 24) showed a very marked oedema of the penis, while only 5 out of 12 remaining rats of the ascorbic acid treated group (12 out of 24) showed slight traces of it.

## Discussion

Any discussion on the possible mechanism underlying the beneficial effect of ascorbic acid on experimental arthritis must be regarded, at this point, as being purely speculative. Nevertheless, our own observations and the following ones: (*a*) that ascorbic acid causes eosinopenia and lymphopenia in guinea-pigs at room temperature (17), (*b*) that it prevents eosinophilia in animals chronically exposed to cold (17), (*c*) that the same substance prevents hypertension in D.C.A. treated animals (7), and (*d*) stimulates the activity of the adrenal cortex as indicated by the fall in adrenal cholesterol content (20) would seem to indicate that vitamin C has some cortisone-stimulating effect. But some of our own previous results (6), which indicated that ascorbic acid was inhibiting the normal hypertrophy of the adrenals normally produced by exposure to cold, seem to contradict the previous hypothesis. It is clear that more experimentation is needed, especially in hypophysectomized animals, for substantiating any plausible hypothesis concerning the action of ascorbic acid on the hypophysis – adrenal axis.

## Summary and Conclusions

*Formaldehyde-induced arthritis*

(1) is considerably aggravated by exposure to cold; the swelling of the injected leg has been measured by displacement of water and the results are statistically highly significant;

(2) is greatly and statistically diminished by ascorbic acid in adult rats, but not in young rats;

(3) is accompanied, in animals exposed to cold, by a severe oedema of the penis appearing much sooner and being far more intense than the one normally encountered after long exposures to cold alone; that effect is strikingly diminished (in intensity and frequency) by large doses of ascorbic acid.

## Résumé

(1). Chez les rats adultes et chez les rats en croissance l'arthrite expérimentale est considérablement aggravée par l'exposition au froid; l'augmentation de volume des pattes injectées fut mesurée par déplacement d'eau, et les résultats sont hautement significatifs.

(2). L'acide ascorbique à fortes doses a un effet préventif qui est statistiquement significatif, mais seulement chez les rats adultes.

(3). L'oedème du pénis, chez les rats traités à la formaline (injection dans la patte gauche) et exposés au froid, est beaucoup plus grave, et apparaît plus rapidement que chez les rats normaux exposés à la même température. L'effet endommageant de la formaline, dans les mêmes conditions, est nettement diminué (en fréquence et en intensité) par l'acide ascorbique.

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# PARALLEL STUDIES OF COMPLEMENT AND COAGULATION

## IV. EFFECT OF CARBON TETRACHLORIDE[1]

By Christine E. Rice, Paul Boulanger, and P. J. G. Plummer


## Abstract

To determine whether liver injury would result in a parallel decline in the complement titer and coagulative properties of the blood, groups of guinea pigs were given series of injections of the liver poison, carbon tetrachloride. Marked fatty degeneration of the liver, a decline in total serum protein and albumin, a decrease in complement activity, and a prolongation of coagulation time was observed in the treated animals. A general relationship was noted between the albumin–globulin ratio and the complement titer of the serum and between the complement titer and the coagulation time of the plasma.


## Introduction

That the liver is the most important site for the manufacture of albumin and various other blood proteins (17, 25, 27), more particularly fibrinogen (6, 7, 8, 9, 13, 19, 29) and the prothrombin complex (15, 22, 24, 26, 28) is generally recognized. Hepatectomy, experimentally produced liver damage, and liver disease are followed by a fall in fibrinogen and prothrombin, the decline corresponding to the degree of injury. After partial hepatectomy, prothrombin may be decreased but fibrinogen remains essentially unchanged (29). Although less attention has been directed toward the rôle of the liver in the maintenance of normal complement levels, a decrease in complement titer has been frequently noted in liver disease (10) and has been produced by phosphorus poisoning (6, 12) and prolonged chloroform anesthesia (11). A simultaneous fall in coagulative activity was recorded in one of these studies (12). It seemed appropriate therefore that the next step in our parallel studies of these two properties of the blood (2, 23) should be an investigation of the effect of liver damage upon each.

Preliminary studies were made with three hepatatoxic agents, chloroform, carbon tetrachloride, and hydrazine sulphate. The results obtained with carbon tetrachloride are described in this paper.

## Methods

Groups of guinea pigs of known weight were injected with varying amounts of carbon tetrachloride. Serial small doses as well as one or two large doses were given since it has been reported that a more acute, massive poisoning may be produced by several small injections than by one large dose (1). The treated animals and a corresponding number of untreated animals of similar weight were bled after the intervals indicated below. The plasma was tested for coagulative activity and the serum for hemolytic complement content.

[1] *Manuscript received December 12, 1950.*

*Contribution from the Division of Animal Pathology, Science Service, Dominion Department of Agriculture, Animal Diseases Research Insitute, Hull, Que.*

Two liver function tests were performed on all sera, the cephalin–cholesterol flocculation test of Hanger and the van den Bergh test for bilirubin. The total protein, globulin, and albumin values of sera from seven normal and 14 injected guinea pigs were also determined. As an additional check on the degree of liver damage, 10 treated animals were killed and their livers, spleens, and kidneys examined for histopathological changes; the findings were compared with those for organs from five normal animals.

*Treatment of Animals*

Five series of guinea pigs, 21 in all, were injected subcutaneously with one to six doses of carbon tetrachloride as follows:

Series I (three guinea pigs)-0.5 ml. injected 11/1; bled 17/1/50.
Series II (three " ")-0.5 ml. injected 18/1; 0.2 ml. 23/1; bled 24/1/50.
Series III (six " ")-0.5 ml. injected 18/1; 0.2 ml. 23/1; bled 26/1/50.
Series IV (three " ")-0.1 ml. injected 2/2, 6/2, and 13/2; bled 20/2/50.
Series V (six " ")-0.1 ml. injected 2/2, 6/2, 13/2, 20/2, 24/2, and 27/2; bled 28/2/50.

None of the animals showed any marked change in weight during the period of observation.

*Methods of Testing*

The complement titrations, prothrombin time determinations, liver function tests, and serum protein estimations were carried out as described in the preceding papers (2, 23).

## Results

That the injection of carbon tetrachloride had produced a considerable degree of liver injury was shown both by liver function tests on serum and by the microscopic appearance of liver sections from representative members of treated guinea pigs.

*Liver Function Tests*

Of the 16 sera tested, 11 reacted in cephalin–cholesterol flocculation tests, 12 gave a positive bilirubin test. All the control sera were negative in both tests.

*Histopathological Examinations*

*Control Animals*

Nothing unusual was noted in the livers of the five control animals examined. There was some slight rarification and hypergranulation of the hepatic cell cytoplasm but these changes were considered to be within the "normal range". The kidneys and spleen appeared normal.

*Treated Animals*

The livers of all 10 animals showed some fatty degeneration, varying in degree from slight to very marked. In some cases it was so extensive as to resemble adipose tissue. Large vacuoles were noted in the majority of liver cells. Some cells were ruptured. In general, the cytoplasm and nucleus were pushed to the periphery but the nucleus itself did not appear to be damaged. The most extensive changes were around the lobular areas but other zones did not escape. Some areas showed peripheral damage. These pathological findings were similar to those previously described as occurring in carbon tetrachloride poisoning (3).

In the kidneys of a number of the treated guinea pigs, the glomeruli were shrunken and the capillary tufts patent. The spleens were normal.

*Serum Protein Analyses*

As would be expected from the results of the cephalin–cholesterol flocculation tests, sera of most of the treated guinea pigs deviated considerably from normal in their protein content. These changes were both quantitative and qualitative. The results of each group of analyses will be considered separately.

*Total Protein*

The seven specimens of normal serum analyzed showed considerable variation in their total protein content: range 7.97 to 9.96, mean 9.28 gm. %. The range for the 14 sera from treated guinea pigs was 5.58 to 8.37, mean 7.45 gm. %. Eight of these 14 sera had total protein values below 7.97 gm. %, the lowest value recorded for the normal group.

*Albumin*

The normal serum albumin values ranged from 4.66 to 5.63, mean 5.33 gm. %. For the 14 treated guinea pigs, the serum albumin range was 2.37 to 4.66, mean 3.73 gm. %; in all but one of these, No. 907, the albumin value was below the minimum for the controls.

*Total Globulin*

In contrast to the decrease in total serum protein and serum albumin, the globulin content of the sera of the treated animals, range 2.93 to 4.92, mean 3.65 gm. %, was relatively comparable with that of the controls, range 3.31 to 4.33, mean 3.95 gm. %.

*Albumin–Globulin Ratio*

As a result of this proportionately greater decrease in albumin than in globulin, the albumin–globulin ratio of sera from treated guinea pigs, 0.51 to 1.26, mean 1.02, was significantly below that of the normal group, range 1.15 to 1.58, mean 1.35. On the basis of electrophoretic analysis, Deutsch and Goodloe (5), reported an albumin–globulin ratio of 1.23 for guinea pig sera.

*Globulin Fractions*

When separated into three fractions by precipitation with 15, 19, and 26 % sodium sulphate, the serum globulins from the two groups of animals showed the differences in their distribution indicated by the mean values below:

| | Fraction I | | Fraction II | | Fraction III | |
|---|---|---|---|---|---|---|
| | Control | Treated | Control | Treated | Control | Treated |
| Grams per 100 ml. | 1.64 | 1.81 | 0.56 | 0.32 | 1.74 | 1.59 |
| % of total protein | 17.6 | 24.4 | 6.2 | 4.1 | 18.7 | 21.4 |
| % of total globulin | 41.3 | 48.8 | 14.6 | 8.4 | 44.1 | 42.8 |

The amount of Fraction I, which may consist largely of gamma-globulin (16), was increased slightly in the treated animals, both in relation to the total protein and to the total globulin. Fraction III on the other hand, which presumably is largely alpha-globulin, was decreased. The values for Fraction II were low throughout which seems in agreement with the results of other investigators who have found guinea-pig serum to be low in beta-globulin (20).

*Complement Titrations*

When the complement titers of the treated animals were compared with those of normal guinea pigs they were found to be significantly lower. Whereas the $K'_{50}$ values, that is amount of complement required for 50 % hemolysis of the standard volume of sensitized sheep red cells, for the 22 control sera ranged from 0.00045 to 0.00088 ml., mean 0.00065 ml., the $K'_{50}$ values for sera of the 19 treated guinea pigs ranged from 0.00069 to 0.0044 ml., mean 0.0016 ml. The mean complement titer of sera from the treated guinea pigs was therefore less than half that of sera from the normal group, 625 as compared with 1538 50% hemolytic units. The plasmas in general had slightly lower complement titers than the corresponding sera. The mean ratio of serum titer: plasma titer for the 21 control specimens was 1.15, and for the 16 bleedings from the injected group 1.14. The treated guinea pigs displayed no greater differences in the relative complement titer of serum and plasma than did the normals.

The titers (expressed in 100% hemolytic units per ml.) of the individual components, C′ 1, C′ 2, C′ 3, and C′ 4, were determined for 13 normal and 12 treated guinea pigs. All four components were found to be lower than normal in the latter group; C′ 4 appeared to be the component most affected.

| Component | Group | Titer (100% hemol. units per ml.) | |
|---|---|---|---|
| | | Range | Mean |
| C′ | Normal | 625 to 1000 | 714 |
| | Treated | 161 to 833 | 333 |
| C′ 1 | Normal | 1440 to 3000 | 2083 |
| | Treated | 625 to 3330 | 1370 |
| C′ 2 | Normal | 670 to 1250 | 833 |
| | Treated | 200 to 1250 | 575 |
| C′ 3 | Normal | 741 to 1330 | 1060 |
| | Treated | 203 to 1670 | 490 |
| C′ 4 | Normal | 2000 to 10,000 | 5850 |
| | Treated | 400 to 3330 | 771 |

*Prothrombin Time Determinations*

In addition to changes in serum proteins and a decline in complement titer, a decrease in the coagulative activity of the plasma of many of the treated guinea pigs was observed. Thus, while the Quick prothrombin time values for the 22 normal plasmas ranged from 21 to 32 sec., mean 26 sec., those of the plasmas of the 19 treated animals ranged from 25 to 75 sec., mean 41 sec. The mean Howell prothrombin times for the control and treated animals were 56 and 91 sec. respectively. The two-stage clotting time for the normal group ranged from 15 to 48 sec., mean 24 sec. For the treated group the range was 22 to 954 sec., mean 210 sec. and median 65 sec. Among the treated animals the least change was observed in the animals given one 0.5 ml. dose of the drug and not bled till six days later (Series I). The greatest change was recorded in the five that received six 0.1 ml. injections over a period of 25 days (Series V). In the conversion phase of the two-stage test, these five sera showed atypical clotting, owing probably to a deficiency in fibrinogen. The differences between the two-stage conversion and clotting times were greater for the plasmas of normal than of treated animals.

Table I lists the serum protein values, complement titers, and coagulation times for seven control and 14 treated guinea pigs together with data on dosage, results of liver function tests, and microscopic findings on liver specimens.

*Relation of Complement Titers to Degree of Liver Damage*

The complement titers of each of the treated guinea pigs was considered in relation to the results of liver function tests and liver pathology. The $K'_{50}$ values of the 11 sera showing positive cephalin–cholesterol flocculation, a reaction indicative of changes in protein content, notably an increase in gamma-globulin in relation to albumin, ranged from 0.0011 to 0.0044 ml., mean 0.0020 ml. (titer = 500 50% hemolytic units per ml.). For the five nonflocculative sera, $K'_{50}$ values varied from 0.00069 to 0.0010 ml., mean

## TABLE I

SERUM PROTEIN VALUES, COMPLEMENT TITERS, AND COAGULATION TIME VALUES FOR BLEEDINGS FROM CONTROL AND CARBON-TETRACHLORIDE-TREATED GUINEA PIGS

| No. of G.P. | Date of bleedings | Series | No. of doses | Liver pathology (fatty degeneration) | | C.C.F.* | V.D.B.** | Total protein, gm. % | Albumin, gm. % | Total globulin, gm. % | Albumin–globulin ratio | % total protein | | | Compl. titer*** | Prothrombin time | | Two-stage | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Distribution | Extent | | | | | | | Fr. I | Fr. II | Fr. III | | Quick | Howell | Conversion | Clotting |
| | 1950 | | | | | | | | | | | | | | | | | | |
| 565 | 26/1 | Controls | 0 | Normal | None | – | – | 9.27 | 5.47 | 3.80 | 1.44 | 16.6 | 8.3 | 16.5 | 1560 | 29 | 62 | 127 | 26 |
| 580 | " | " | 0 | " | | – | – | 9.17 | 5.63 | 3.54 | 1.58 | 16.6 | 4.7 | 17.2 | 1540 | 28 | 78 | 120 | 20 |
| 567 | " | " | 0 | " | | – | – | 8.78 | 4.74 | 4.04 | 1.15 | 18.8 | 10.2 | 17.0 | 1590 | 28 | 78 | 138 | 29 |
| 571 | 28/2 | " | 0 | " | | – | – | 9.96 | 5.63 | 4.33 | 1.30 | 20.0 | 4.4 | 19.2 | 1140 | 26 | 55 | 162 | 48 |
| 559 | " | " | 0 | " | | – | – | 7.97 | 4.66 | 3.31 | 1.41 | 14.2 | 7.6 | 19.6 | 2170 | 32 | 56 | 129 | 24 |
| 574 | " | " | 0 | " | | – | – | 9.96 | 5.63 | 4.33 | 1.30 | 17.1 | 4.0 | 22.4 | 1610 | 22 | 54 | 135 | 30 |
| 575 | " | " | 0 | " | | – | – | 9.86 | 5.53 | 4.33 | 1.28 | 20.0 | 4.4 | 19.2 | 1920 | 27 | 67 | 126 | 25 |
| 563 | 24/1 | Treated | 2 | Diffuse | Marked | + | + | 8.37 | 4.49 | 3.88 | 1.15 | 25.2 | 4.3 | 16.9 | 227 | 61 | 109 | 848 | 408 |
| 570 | 26/1 | " | " | " | " | + | + | 7.57 | 4.23 | 3.34 | 1.26 | 24.8 | 1.7 | 17.8 | 667 | 30 | 69 | 380 | 67 |
| 577 | " | " | " | " | " | + | + | 6.00 | 3.07 | 2.93 | 1.05 | 24.0 | 2.2 | 22.5 | 361 | 44 | 117 | 480 | 270 |
| 562 | " | " | " | " | " | + | + | 7.58 | 3.84 | 3.74 | 1.03 | 24.8 | 1.7 | 22.9 | 476 | 44 | 139 | 420 | 262 |
| 572 | " | " | " | " | Moderate | + | – | 7.57 | 3.97 | 3.60 | 1.10 | 20.4 | 2.1 | 24.4 | 667 | 33 | 92 | 370 | 65 |
| 579 | " | " | " | " | " | + | + | 6.97 | 3.71 | 3.26 | 1.13 | 24.8 | 3.2 | 18.9 | 500 | 32 | 80 | 376 | 70 |
| 581 | " | " | " | Zonal | Slight | + | ± | 7.97 | 4.23 | 3.74 | 1.12 | 23.4 | 9.1 | 22.9 | 1000 | 47 | 116 | 328 | 58 |
| 904 | 20/2 | " | 3 | " | " | – | + | 5.58 | 2.49 | 3.09 | 0.88 | 24.3 | 6.0 | 21.1 | 1180 | 30 | 68 | 155 | 35 |
| 578 | " | " | " | " | Moderate | – | – | 6.58 | 3.20 | 3.38 | 0.95 | 21.7 | 1.8 | 22.9 | 1200 | 29 | 62 | 158 | 31 |
| 569 | 28/2 | " | 6 | " | Marked | + | + | 8.37 | 4.48 | 3.89 | 1.15 | 21.7 | 5.8 | 16.7 | 1000 | 44 | 97 | 430 | 320 |
| 907 | " | " | " | | | + | + | 8.37 | 4.66 | 3.71 | 1.25 | 21.5 | 6.6 | 22.1 | 588 | 75 | 125 | 410 | 262 |
| 585 | " | " | " | | | + | + | 7.97 | 3.97 | 4.00 | 0.99 | 25.2 | 6.3 | 27.1 | 667 | 50 | 92 | 660 | 569 |
| 909 | " | " | " | | | + | + | 8.37 | 3.45 | 4.92 | 0.70 | 34.7 | 9.0 | 22.5 | 435 | 73 | 158 | 760 | 954 |
| 911 | " | " | " | | | + | + | 6.97 | 2.37 | 3.60 | 0.51 | 24.8 | 1.7 | 21.2 | 919 | 41 | 105 | 420 | 420 |
| Mean | | Untreated | | | | | | 9.28 | 5.33 | 3.95 | 1.35 | 17.6 | 6.2 | 18.7 | 1647 | 27 | 64 | 134 | 29 |
| Mean | | Treated | | | | | | 7.45 | 3.73 | 3.65 | 1.02 | 24.4 | 4.1 | 21.4 | 706 | 45 | 102 | 435 | 285 |

* *Cephalin–cholesterol flocculation test.*

** *van den Bergh test for bilirubin.*

*** *Complement titers expressed in 50% hemolytic units.*

0.00062 ml. (mean titer = 1613 units per ml.). The 12 sera which gave a positive bilirubin test, had $K'_{50}$ values of 0.0010 to 0.0032 ml., mean 0.0016 ml. (mean titer = 625 units per ml.), whereas the four that were negative for bilirubin had a range of $K'_{50}$ values of 0.00069 to 0.0015 ml., mean 0.00094 ml. (titer = 1064 units per ml.).

A relationship was also apparent between complement titers and the distribution and extent of the fatty degeneration observed in the liver.

| Fatty degeneration | | No. of sera | Complement titer (50% hemolytic units per ml.) | |
|---|---|---|---|---|
| Distribution | Extent | | Range | Mean |
| Zonal | Slight | 2 | 1000 to 1176 | 1088 |
| Zonal | Moderate | 1 | 1204 | 1204 |
| Zonal | Marked | 1 | 1000 | 1000 |
| Diffuse | Moderate | 2 | 500 to 667 | 584 |
| Diffuse | Marked | 4 | 227 to 667 | 377 |
| Controls | — | 22 | 1136 to 2220 | 1538 |

*Relationship of Prothrombin Time to Degree of Liver Damage*

A similar relationship was demonstrated between the prothrombin time values as determined by the Quick, Howell, and two-stage methods and the degree of liver damage produced by the injection of carbon tetrachloride. For example, the 11 animals whose sera gave positive cephalin–cholesterol flocculation tests had two-stage clotting times of 67 to 954 sec., mean 333 sec., whereas the five with negative sera had a range of 29 to 58 sec., mean 41 sec. Plasma of the 12 animals showing the presence of bilirubin in their sera had two-stage clotting times of 35 to 954 sec., mean 308 sec.; the four with bilirubin-negative sera had clotting times of 29 to 65 sec., with a mean of 44 sec. The apparent relationship between two-stage clotting time values and liver pathology is indicated by the following summary:

| Fatty degeneration | | No. of plasmas | Two-stage clotting time (sec.) | |
|---|---|---|---|---|
| Distribution | Extent | | Range | Mean |
| Zonal | Slight | 2 | 35 to 48 | 42 |
| Zonal | Moderate | 1 | 31 | 31 |
| Zonal | Marked | 1 | 320 | 320 |
| Diffuse | Moderate | 2 | 65 to 70 | 68 |
| Diffuse | Marked | 4 | 67 to 408 | 252 |
| Controls | — | 22 | 15 to 48 | 24 |

*Comparison of Complement Titers and Serum Protein Values*

It seemed convenient in studying the relationship of complement activity to serum protein content, to divide the 21 sera on which protein estimations

had been made into three groups on the basis of complement titer. The first group (with titers over 1000 units per ml.) which included all of the normal sera and sera from two treated animals (Series II and III) had the highest mean total protein and albumin values and the highest mean albumin–globulin ratio (Table II). The mean total protein and albumin values for the second

TABLE II

COMPARISON OF COMPLEMENT TITER WITH SERUM PROTEIN VALUES

| Complement titer,* units/ml. | No. of sera | Total protein, gm. % | | | Albumin, gm. % | | | Albumin–globulin ratio | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Range | Mean | σ | Range | Mean | σ | Range | Mean | σ |
| >1000 | 9 | 5.58 to 9.96 | 8.57 | 1.66 | 2.49 to 5.53 | 4.77 | 1.26 | 0.88 to 1.58 | 1.25 | 0.20 |
| 500 to 1000 | 8 | 6.97 to 8.37 | 7.72 | 0.58 | 2.37 to 4.66 | 3.95 | 0.75 | 0.51 to 1.26 | 1.06 | 0.25 |
| <500 | 4 | 6.00 to 8.37 | 7.58 | 1.29 | 3.07 to 4.49 | 3.71 | 0.69 | 0.70 to 1.15 | 0.98 | 0.23 |

* *Expressed in 50% hemolytic units.*

(titers 500 to 1000 units per ml.) and the third (titers less than 500 units per ml.) were somewhat lower, but not significantly so on statistical evaluation. The difference between the mean albumin–globulin ratios for the first and third groups, 0.27, was 2.0 times the standard error. The mean total globulin values for the three groups were relatively comparable.

Differences were evident however on examination of the relative amounts of the three globulin fractions in sera of high, and low complement titers (Table III). Although the mean amount per ml. of Fraction I was only

TABLE III

COMPARISON OF COMPLEMENT TITER WITH MEAN SERUM GLOBULIN VALUES

| Complement titer,* units ml. | No. of sera | Globulin, gm. % | | | | % total protein | | | % total globulin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Fr. I | Fr. II | Fr. III | Fr. I | Fr. II | Fr. III | Fr. I | Fr. II | Fr. III |
| >1000 | 9 | 3.79 | 1.60 | 0.54 | 1.65 | 19.0 | 6.5 | 19.5 | 42.1 | 14.5 | 43.4 |
| 500 to 1000 | 8 | 3.64 | 1.87 | 0.31 | 1.60 | 24.3 | 4.0 | 20.8 | 49.5 | 7.8 | 42.8 |
| <500 | 4 | 3.87 | 1.88 | 0.29 | 1.69 | 24.8 | 3.6 | 22.4 | 49.3 | 6.9 | 43.9 |

* *Expressed in 50% hemolytic units.*

slightly higher in the third than in the first titer group, this difference was found to be statistically significant when considered in relation to total serum protein and globulin. Fraction II on the other hand was lower both in actual amount and in proportion to total protein and total globulin. Fraction III was present in relatively comparable amounts in sera of all three complement titer groups; its proportion in total globulin was also similar.

*Comparison of Coagulation Times and Serum Protein Values*

The two-stage clotting times were selected for this analysis for two reasons. In the first place, they had indicated the greatest differences between the treated and the control groups. In the second place, like the serum protein values, they do not reflect variation in the amount of fibrinogen, since an excess of this component is added in this technique.

The first group of specimens with two-stage clotting times of 18 to 30 sec. had the highest mean total serum protein content, as well as the highest albumin–globulin ratios (Table IV). This group consisted entirely of

TABLE IV

COMPARISON OF TWO-STAGE CLOTTING TIME OF PLASMA WITH SERUM PROTEIN VALUES

| Clotting time, sec. | No. of specimens | Total protein, gm. % | | | Albumin, gm. % | | | Albumin–globulin ratio | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Range | Mean | σ | Range | Mean | σ | Range | Mean | σ |
| 18 to 30 | 6 | 7.97 to 9.96 | 9.17 | 0.80 | 4.66 to 5.63 | 5.28 | 0.60 | 1.15 to 1.58 | 1.36 | 0.16 |
| 31 to 48 | 3 | 5.58 to 9.96 | 7.37 | 2.80 | 2.49 to 5.63 | 3.77 | 2.01 | 0.88 to 1.30 | 1.04 | 0.27 |
| 58 to 78 | 4 | 6.97 to 7.57 | 7.52 | 0.46 | 3.71 to 4.23 | 4.04 | 0.28 | 1.10 to 1.26 | 1.15 | 0.08 |
| 256 to 954 | 8 | 6.00 to 8.37 | 7.75 | 0.92 | 2.37 to 4.66 | 3.79 | 0.85 | 0.51 to 1.25 | 0.98 | 0.26 |

normal plasmas. One normal specimen was included in the second group of three specimens. The third and fourth groups were made up of bleedings from treated guinea pigs. The mean total serum protein and albumin values and albumin–globulin ratios for these two groups were significantly lower than those of the first (differences 2.6 to 4.4 times the standard error). The total serum globulin values were closely comparable for the four categories (Table V). The amount of Fraction I was slightly higher in the fourth than

TABLE V

COMPARISON OF TWO-STAGE CLOTTING TIME OF PLASMA WITH MEAN SERUM GLOBULIN VALUES

| Clotting time, sec. | No. of specimens | Globulin, gm. % | | | | % total protein | | | % total globulin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Fr. I | Fr. II | Fr. III | Fr. I | Fr. II | Fr. III | Fr. I | Fr. II | Fr. III |
| 18 to 30 | 6 | 3.89 | 1.59 | 0.59 | 1.72 | 17.2 | 6.5 | 18.7 | 40.6 | 15.3 | 44.1 |
| 31 to 48 | 3 | 3.60 | 1.63 | 0.44 | 1.53 | 22.6 | 6.5 | 21.1 | 45.2 | 12.7 | 42.1 |
| 58 to 78 | 4 | 3.49 | 1.79 | 0.16 | 1.54 | 23.7 | 2.2 | 20.6 | 51.2 | 4.5 | 44.2 |
| 256 to 954 | 8 | 3.83 | 1.91 | 0.37 | 1.68 | 24.9 | 4.7 | 21.7 | 48.5 | 9.0 | 42.6 |

in the first group, 0.32 gm. %, or 3.0% of the total protein; whereas the values for Fraction II were somewhat lower, those for Fraction III were closely comparable.

*Comparison of Complement Titers and Prothrombin Times*

The group of 27 specimens with complement titers of over 1000 units per ml., had clotting times within the "normal" range, both as determined by the Quick and by the two-stage method (Table VI). Of the 14 specimens with

TABLE VI

COMPARISON OF COMPLEMENT TITER OF SERUM WITH QUICK PROTHROMBIN AND TWO-STAGE CLOTTING TIMES OF PLASMA

| Complement titer,* units/ml. | No. of specimens | Quick prothrombin time, sec. | | | Two-stage clotting time, sec. | | |
|---|---|---|---|---|---|---|---|
| | | Range | Mean | $\sigma$ | Range | Mean | $\sigma$ |
| >1000 | 27 | 21 to 30 | 26 | 5.2 | 15 to 50 | 26 | 9.3 |
| 500 to 1000 | 9 | 30 to 75 | 42 | 15.2 | 30 to 569 | 207 | 207 |
| < 500 | 5 | 34 to 73 | 51 | 17.4 | 59 to 954 | 391 | 304 |

* *Expressed in 50% hemolytic units.*

complement titers of 252 to 1000 units per ml., eight had two-stage clotting times of over 260 sec.; only one had a clotting time within the normal range, 30 sec. Although the prolongation of the Quick prothrombin times was less marked, nevertheless the differences between the mean values of the first and second, and the first and third groups were statistically significant, 3.1 and 3.3 times the standard error.

## Discussion

Thus, whereas our dicumarol experiment had demonstrated no relationship between prothrombin time values and complement titers (2), this and the preceding study with animals on a low-protein diet (23) have shown a definite prothrombin decline to be accompanied by a significant drop in whole complement titer. For example, four specimens from dicumarol-treated guinea pigs with two-stage clotting times of over 600 sec. had a mean complement titer of 1645 50% hemolytic units per ml., a "normal" value, while seven specimens from guinea pigs in which an extensive liver damage had been produced, with two-stage clotting times of 262 to 947 sec., had a mean complement titer of 461 units per ml., about one-third of the mean titer for normal, untreated animals. Eight specimens from animals on a low-protein diet with slightly prolonged clotting times, 50 to 99 sec., showed a mean complement titer of 945 units per ml., a value just below the minimum "normal" range.

As discussed in preceding papers in this series (2, 23), certain previous investigators have suggested that prothrombin and complement "midpiece" are the same entity. The results of our dicumarol experiment would seem either to disprove this hypothesis or to indicate that the alteration produced in the prothrombin-midpiece complex by the drug extended only to its activity in the coagulation system and not to its properties as a complement

component. In both of our experimental studies in which a corresponding decrease in prothrombin and complement titer was induced, a concomitant drop in total serum protein was observed. This was effected in the one case by reducing the protein intake of the animals, in the other case by damaging the liver to such an extent that its protein synthesis was greatly impaired. The apparent correlation between the changes in complement and coagulative properties demonstrated in these last two studies might be interpreted therefore, either as indicating that a common protein component (prothrombin-midpiece complex) had been reduced or that the protein components of two independent systems had been affected to a relatively similar degree. However, prothrombin as well as first and second components of complement (21) make up such a very small fraction of the total proteins that such a change would not be readily detected by the fractionation methods employed. In passing it should also be pointed out that part of the observed fall in complement titer may have been traceable to the increased proportion of gamma-globulin (14, 18), for it is well recognized that this globulin fraction is highly anticomplementary (4).

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# PAPER CHROMATOGRAPHY OF CASEIN AND RENNIN[1]

By Kenneth W. McKerns


## Abstract

Purified rennins of very high activity were prepared. The amino acid composition of the hydrolyzates of these preparations was studied by paper chromatography. Several fractions were obtained from the purified rennins by paper chromatography showing strong enzyme activity which was comparable to the density of their color development with a benzidine reagent. Whether these fractions represent distinct molecular species is not known as yet. The active enzyme was separated from the inert material of a crude commercial rennet powder by chromatography. The action of rennin on milk and casein was also studied by chromatographic techniques. It would appear that rennin causes an unfolding and breaking of the alpha casein molecule with the appearance of several large polypeptides. No free amino acids were detected after rennin action on casein.


Even though the mechanism of the clotting of milk and casein by rennet has been the subject of much study, little is known of the exact nature of paracasein formation. Berridge (1) suggested that the formation of paracasein represents an unfolding of the casein molecule due to rennin action. The soluble paracasein in the presence of calcium forms the familiar clot of insoluble calcium paracaseinate. Casein is a variable mixture of at least two components (7). The larger alpha component is the one acted on by rennin. Warner (9), who prepared pure alpha and beta casein, found a tendency for alpha casein to split spontaneously into two fractions. Nitschmann and Lehmann (8) consider the action of rennin on alpha casein as an acceleration of this tendency since they found a change in the electrophoretic pattern of casein after rennin action.

This paper represents a preliminary study of casein and rennin by proper chromatographic techniques.

## Materials and Methods

Purified rennins of high activity were prepared according to the method of Hankinson (6). Difficulty was experienced in obtaining a crystalline product but amorphous precipitates, giving activities of the order of $10^7$, were readily obtained. The precipitates were then dissolved in water adjusted to pH 6.5 and lyophilized. The enzyme activities were measured at 37° C. using powdered skim milk dissolved in normal acetate buffer of pH 5.0. The reaction time was 10 min. The casein used in the chromatography was a calcium free British Drug House preparation filtered before use and adjusted to pH 6.5 by adding normal acetate buffer of pH 5.0.

The chromatography on Whatman No. 1 filter paper was carried out using the capillary ascent technique of Williams and Kirby (10). Hemin was used as a marker for the protein according to the methods of Franklin and Quastel

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*Contribution from the Research Laboratories, Canada Packers, Toronto.*

(4, 5). A 3% solution of crystalline hemin dissolved in distilled water with the aid of a little sodium bicarbonate was generally used in the proportions of 0.01 ml. hemin solution to 0.5 ml. of 2% protein solution. Organic salts and buffer solutions of various concentrations and pH were used as developing solvents. The best separations were achieved using $M/10$ sucrose in the first dimension and $M/10$ sodium potassium tartrate in the second dimension. The dried chromatograms were streaked with freshly prepared benzidine solution* and photographed before the background color appeared (5).

To locate the active enzyme fractions, two chromatograms were run under the same conditions, one without the hemin marker. After benzidine was applied to the chromatogram containing the hemin, it was used as a guide in cutting out the various fractions. These cut out portions were then covered with normal acetate buffer of pH 5.0 in a small test tube and an equal volume of whole milk added. The rennin activities of the various fractions could then be compared.

Acid hydrolyzates of the purified rennins were prepared by refluxing them in 6*N* hydrochloric acid for 24 hr., evaporating to dryness in vacuo at 45° C., and dissolving the residue in 75% (v/v) ethanol. The slightly acid alcohol solutions of the amino acids obtained were spotted on 15 in. squares of filter paper which were stapled in the form of cylinders and developed with various organic solvents. The dried chromatograms were then sprayed with 0.1% ninhydrin in *n*-butanol saturated with water according to the technique of Consden, Gorden, and Martin (2).

## Results and Conclusions

The chromatograms of the acid hydrolysis of pure rennins are shown in Figs. 1 and 2. The following developing solvents gave the best separations:—ethanol : butanol : 10*N* ammonium hydroxide (60 : 20 : 20); butanol saturated with 5 × diluted glacial acetic acid; equal parts collidine and lutidine saturated with water. The $R_F$ values of all the pure amino acids dissolved in 75% (v/v) ethanol were determined separately in each developing solvent and two dimensional "maps" were plotted for various combinations of developing solvents. A tentative identification of amino acids in the chromatograms of the hydrolyzates could then be attempted. A corresponding mixture of pure amino acids was then chromatographed to check the identification. Fig. 1 shows the pattern obtained with butanol–acetic in the first dimension and collidine–lutidine in the second. Fig. 2 shows a rennin hydrolyzate chromatographed with ethanol–butanol–ammonia and butanol–acetic. Elution of the spots and further hydrolysis following the method of Consden, Gorden, and Martin (3) did not change the amino acid pattern. An alkaline hydrolysis has not yet been attempted so it is not known whether tryptophane is present. No threonine, norleucine, or histidine was found. Isoleucine was not identified.

* *Equal volumes of saturated alcoholic benzidine and 3% hydrogen peroxide (diluted from 30% superoxol) made acid with glacial acetic acid.*

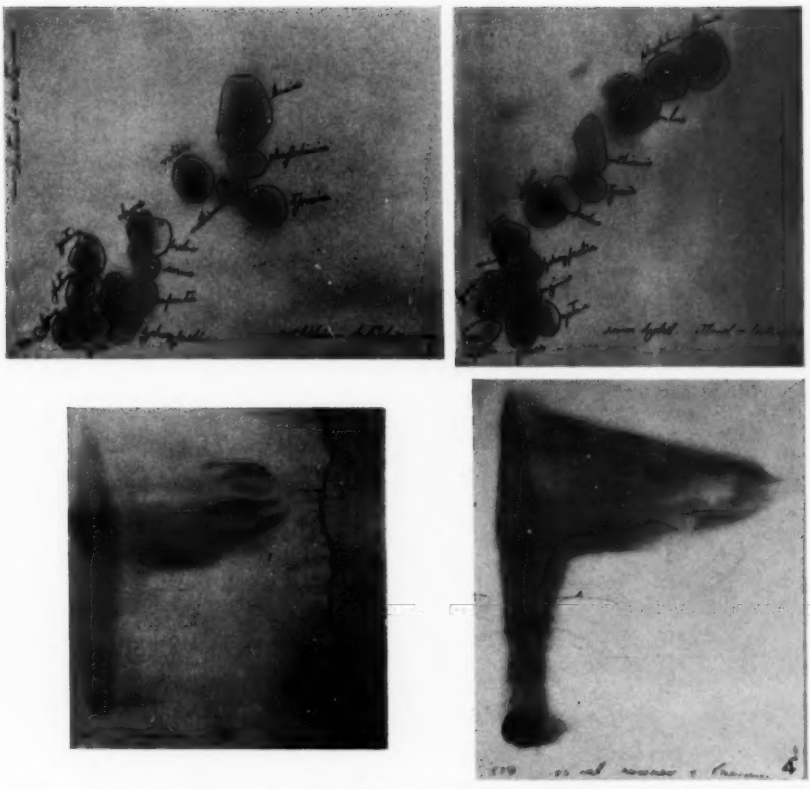


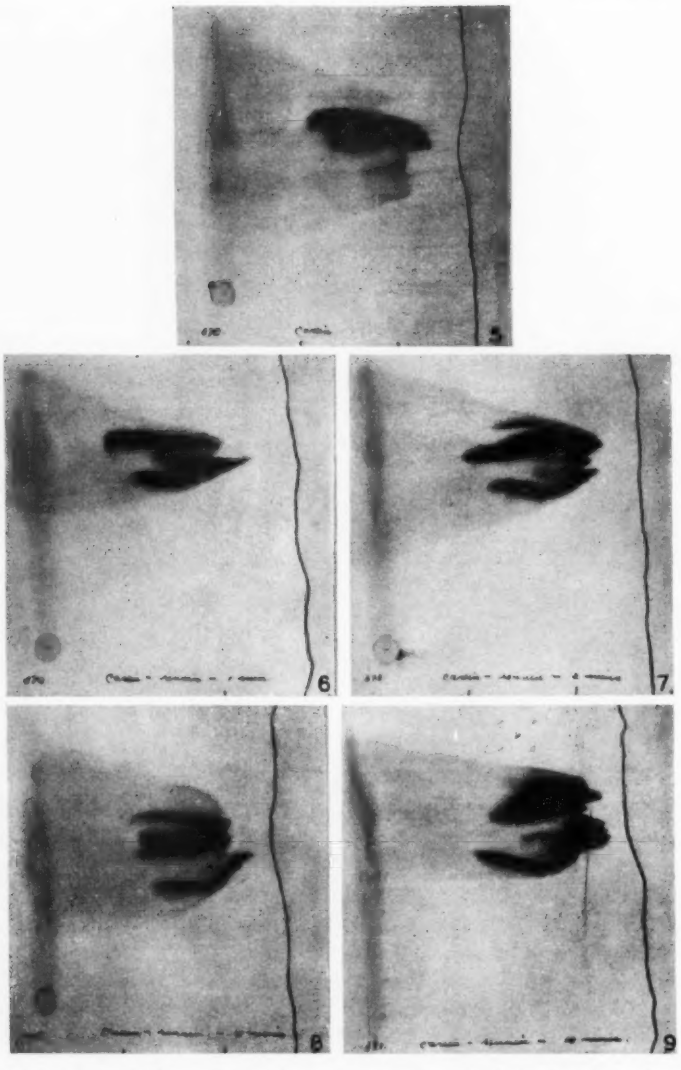
FIG. 1. Acid hydrolyzate of rennin chromatographed in butanol–acetic followed by collidine–lutidine.

FIG. 2. Acid hydrolyzate of rennin chromatographed in ethanol–butanol–ammonia followed by butanol–acetic.

FIG. 3. Chromatogram of 0.04 ml. of a 1% solution of purified rennin in sucrose and tartrate solutions.

FIG. 4. Chromatogram of 0.05 ml. of a 6% solution of crude rennin in sucrose, tartrate solutions.

PLATE II



FIGS 5-9. The change in the chromatogram of casein during rennin action.

PLATE III

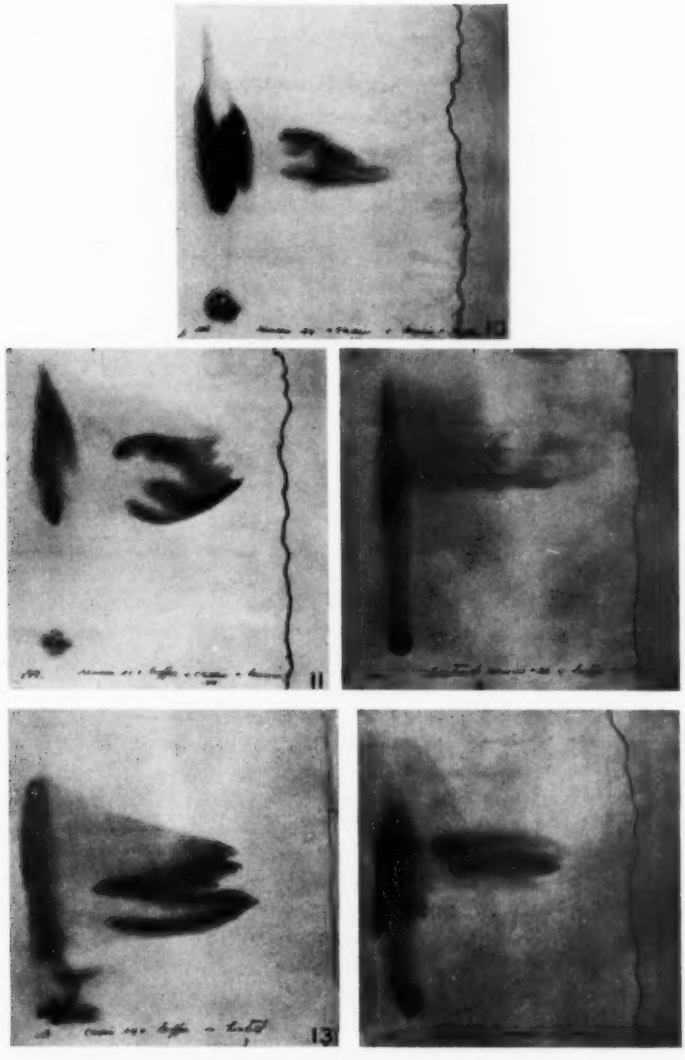


FIG. 10. Chromatogram of rennin action on casein using an excess of enzyme.
FIG. 11. Chromatogram of rennin action on casein using an excess of enzyme.
FIG. 12. Chromatogram of rennin denatured by boiling.
FIG. 13. Chromatogram of partially denatured casein.
FIG. 14. Chromatogram of casein and denatured rennin.

The chromatogram of a 1% solution of rennin in water at pH 6.5 is shown in Fig. 3. Several fractions appear when sucrose is used as the developing solvent in the first dimension followed by tartrate. All the fractions showed rennin activity, the greatest enzyme activity being found in the two major fractions. It is not known as yet whether the various fractions represent different naturally occurring molecular species of rennin. A comparison of the hydrolyzates of the fractions with the original rennin hydrolyzates will reveal any difference in the amino acid composition.

The chromatogram of a commercial rennet powder having 1/20 the rennin activity of the purified material is shown in Fig. 4. The powder was dissolved in water and adjusted to pH 6.5 with 0.1*N* sodium hydroxide. It was necessary to use 0.05 ml. of a 6% solution to obtain a satisfactory chromatogram. A great deal of the material did not move from the starting point. Most of the rennet activity was found in the largest component. No activity was detected in the first dimensional streak or at the point of origin. It would appear that a separation of the active rennin from the inert material had been achieved.

The change in the chromatographic appearance of casein during rennin action is shown in Figs. 5-9. Two ml. of a 4% casein solution, adjusted to pH 6.5 with normal acetate buffer of pH 5.0, was dispensed into a small test tube at 25° C., containing 0.04 ml. of 3% hemin solution. To this was added 0.01 ml. of 0.84% rennin solution. Then 0.02 ml. of the enzyme–substrate mixture was spotted at one minute intervals on 8-in. squares of Whatman No. 1 filter paper stapled in the form of cylinders. The protein spots were dried quickly in a warm current of air and chromatographed with sucrose in the first dimension and tartrate in the second. The rennin and the casein were not denatured by the warm air drying. In fact, fully denatured proteins will not chromatograph with this technique. Thus it is quite likely that some enzyme action proceeds during the chromatographing. This is unimportant provided the rennin concentration is low enough to allow the progressive changes in the casein to be followed. With the small quantity of enzyme used no spots due to rennin itself are revealed on the benzidine treated chromatogram. It can be seen in the photographs that there was considerable unfolding of the casein mixture with the appearance of several new protein fractions. The chromatography of rennin–casein mixtures in several organic solvent solutions did not show any amino acids appearing due to rennin action. The action of rennin on pure alpha casein will be reported subsequently.

The effect of a great excess of enzyme is shown in Fig. 10 and Fig. 11. Enzyme action had not gone to completion. Strong rennin activity was obtained from what appeared to be an enzyme substrate complex in the first dimensional streak in Fig. 10. The excess rennin was mostly confined to the upper fraction in Fig. 11. Fig. 12 shows 0.03 ml. from a 1% rennin solution in water which had been denatured by boiling. The slight movement in the first dimension was mostly due to the hemin which was applied to the

paper after the rennin. Compare this with Fig. 3. A boiled solution of casein also showed little movement. The chromatogram of casein which had been partially denatured by drying the spotted paper in an oven at 100° C. for five minutes is shown in Fig. 13. The spreading of the pattern must be due to the unfolding of the casein molecules. This has an appearance similar to the photograph of the initial phase of rennin action shown in Fig. 6. No change in the casein is shown in Fig. 14 when a mixture of casein and denatured rennin was chromatographed.

## Discussion

Paper chromatography is capable of detecting subtle changes in protein configuration. This is well demonstrated in the chromatograms of rennin action on casein. In the case of rennin the enzyme retains its activity after chromatography. This is not true of some especially labile enzymes such as thrombin. The fact that active rennin may be separated from a crude rennet preparation is especially interesting. It suggests that specific absorbents may be of use in the concentration of rennin in a large scale purification.

## Acknowledgments

The preliminary aspects of this work were carried out under the direction of Dr. J. H. Quastel, Director of the Laboratories of the Research Institute of the Montreal General Hospital. The author also wishes to thank Mr. A. E. Franklin of the same Institute for helpful advice.

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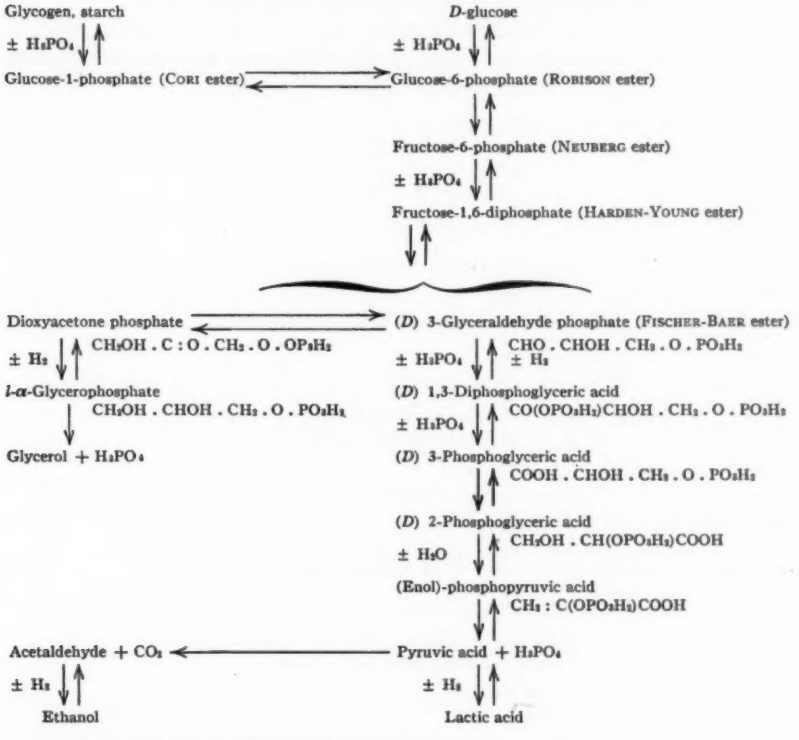
# MECHANISMS OF GLYCOLYSIS AND FERMENTATION[1]

By O. Meyerhof[2]

Recently a well known British biochemist stated that our enzymatic schemes of metabolic intermediaries were in bad need of being confirmed by *in vivo* experiments (1). For this purpose, he found that citric acid accumulates in rats after poisoning with fluoroacetate as it was supposed to do according to the tricarboxylic acid cycle of Krebs. A similar observation was made independently by Kalnitsky (3). Personally, I have never doubted before this gratifying demonstration that the tricarboxylic acid cycle as found by

Scheme 1. Scheme of fermentation and glycolysis.




[1] *Manuscript received November 14, 1950.*

*Contribution from the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia, Pa.*

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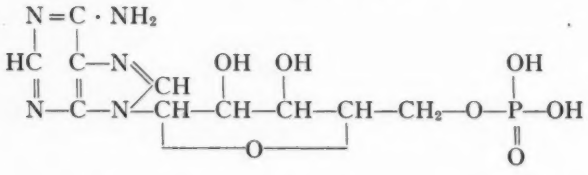
[2] *Research Professor.*

enzymatic experiments would apply to the oxidative metabolism *in vivo*. But when I restrict myself to the glycolytic breakdown to lactic acid, I may say that long ago there was already overwhelming evidence that this metabolism passes *in vivo* through the same intermediary stages as *in vitro*. Most of this evidence was collected from muscle but some also from other organs. Likewise, we have known for a long time that this breakdown is closely related to sugar fermentation in various forms. Here the outstanding example is the alcoholic fermentation of yeast. Only as a reminder do I show you here this old scheme of intermediaries of glycolysis and fermentation as it was developed more than ten years ago.

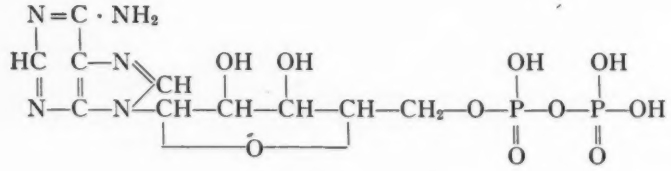
To this scheme of course have to be added the various enzyme and coenzyme systems. Because these enzyme and coenzyme systems act simultaneously on various phases, there is an interrelation of speed depending not only on the amounts of enzymes and coenzymes but also on their availability in their special stages. One of the most important regulators is the phosphorylating coenzyme, the adenylic system, which I show in Scheme 2.

Scheme 2.

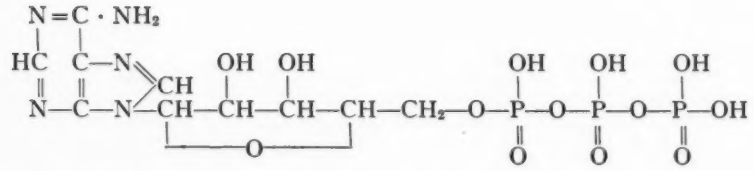
Adenylic system



Adenylic acid (adenosine-5′-monophosphoric acid)



Adenosinediphosphoric acid



Adenylpyrophosphate (adenosinetriphosphoric acid)

If, for instance, there are present both phosphate donors and phosphate acceptors, it makes a great difference whether the enzymes for acceptance and release of phosphate are in balance or whether they are not. If there is too much enzyme activity taking away the phosphate from ATP,* the reaction slows down because the concentration of ATP relative to ADP and AMP becomes too low. On the other hand, if there is too much activity in transphosphorylating the phosphate group to the adenylic system, all of it is transformed into ATP and the reaction stops because it no longer can accept new phosphate groups. Overlooking this point has led to numerous misunderstandings in regard to the phosphorylating cycle *in vitro* and *in vivo*.

Let me first explain this fact somewhat more closely in regard to the cell-free fermentation of yeast. If we start with one glucose molecule, we have first two "transphosphorylating" steps in which ATP reacts with sugar to give hexose diphosphate (HDP) (No. 1 and 5 in Scheme 3).

Scheme 3. Initial reactions of free sugar in fermentation and glycolysis.

(Hexokinase (yeast, brain))

1. Glucose + ATP $\longrightarrow$ glucose-6-phosphate + ADP
2. Fructose + ATP $\longrightarrow$ fructose-6-phosphate + ADP

(Fructokinase (muscle, liver))

3. Fructose + ATP $\longrightarrow$ fructose-1-phosphate + ADP

(Phosphohexoisomerase)

4. Glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate

(Phosphohexokinase (= phosphofructokinase))

5. Fructose-1-phosphate + ATP $\longrightarrow$ HDP + ADP
   Fructose-6-phosphate + ATP $\longrightarrow$ HDP + ADP

Then as shown in the Scheme 4, we have two further steps in which inorganic phosphate is taken up without the interference of the adenylic system and in which the energy stems from the oxidation–reduction. These four phosphate

* *Abbreviations used in this paper: ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate; ATP-ase = adenosine triphosphatase splitting the first labile P group of ATP; HDP = hexose diphosphate (fructose-1,6-diphosphate); DPN = diphosphopyridine nucleotide or cozymase (CoI); Pyro P = pyrophosphate group hydrolyzed in seven minutes by N hydrochloric acid.*

Scheme 4. Oxidative reaction of fermentation.

Stoichiometric-coupled reaction: (Meyerhof, Ohlmeyer, Kiessling, 1937-38)

(d) 3-Glyceraldehyde phosphate + DPN + adenosine diphosphate + $H_3PO_4$ $\rightleftharpoons$

(d) 3-Phosphoglyceric acid + $DPNH_2$ + adenosine triphosphate

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Warburg, Christian's explanation of the stoichiometric-coupled reaction (1939)

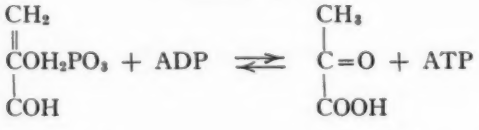
A. (d) 3-Glyceraldehyde phosphate + $H_3PO_4$ $\rightleftharpoons$ 1,3-diphosphoglyceraldehyde

B. 1,3-Diphosphoglyceraldehyde + DPN $\rightleftharpoons$ 1,3-diphosphoglyceric acid + $DPNH_2$

C. 1,3-Diphosphoglyceric acid + adenosine diphosphate $\rightleftharpoons$ 3-phosphoglyceric acid + ATP

groups per mole of hexose are then contained in two molecules of 1,3-diphosphoglyceric acid. In the dephosphorylating reactions these four P groups are released again, all four forming high energy P groups of ATP. The first two stem from carboxylphosphate of the two moles of diphosphoglyceric acid. (Reaction C of Scheme 4.) The next two, as shown in the Scheme 5, derive from two moles of phosphopyruvate.

Scheme 5.



Phosphopyruvate Pyruvate

Now in sum we have dephosphorylated two energy rich phosphate groups of ATP for building up one HDP but we have rephosphorylated four ATP groups. This is the underlying reason for the so-called Harden–Young equation and the kinetics expressed in the Harden–Young effect. Because the amount of the adenylic system is restricted, the excess ATP formed transfers its phosphate to glucose, and for every molecule of HDP split, two more HDP are formed which leads to an autocatalytic increase of HDP. This is also a model of growth of a biological substance by means of a metabolic reaction. Actually, we have to inoculate a trace of HDP in an enzyme extract containing glucose to start fermentation. From then on HDP "grows" autocatalytically as long as sugar and phosphate are present.

Is this now also the condition in the living cells? We can say yes and no. Yes, in so far as the same intermediaries are formed. No, in so far as the distribution and the activity of the enzymes concerned are different.

In the balance which I have discussed so far we have left out one important enzyme, the ATP-ase. If there would be enough ATP-ase, which irreversibly splits the ATP back to ADP, there would not occur the extra phosphorylation of HDP. Actually, it is the lack of ATP-ase in the maceration juice of yeast and even in the dried yeast preparations which is the main condition that the Harden–Young equation shows up. I have recently prepared a type of dried yeast by carefully avoiding autolysis, which has preserved its ATP-ase to a large extent, but is permeable to phosphate and coenzymes. Such a yeast shows no Harden–Young effect, sugar ferments until exhaustion with constant speed. However, if we inhibit the ATP-ase without inhibiting any other enzymes we immediately get the Harden–Young effect. I show this

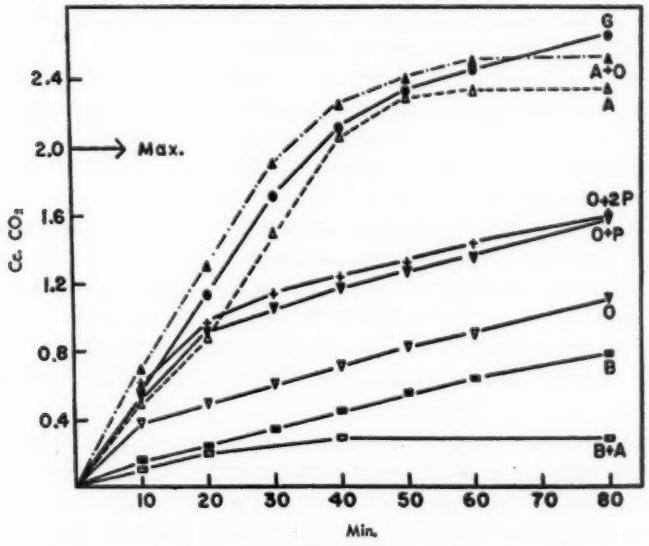



FIG. 1. Manometric measurement of fermentation. Fermentation of glucose by quickly dried yeast; 8 mgm. of glucose added corresponding to 2.0 cc. of $CO_2$. Gas space, $N_2$ + 5% $CO_2$. All samples contain 0.75 cc. of yeast suspension (61.5 mgm., dry weight) made up to 1.0 cc. by various additions in the main compartment. At zero time 0.6 cc. of a solution is tipped in from the side arm with the following ingredients: 8 mgm. of glucose, HDP containing 30 $\gamma$ of P, 400 $\gamma$ of DPN, ATP with 65 $\gamma$ of pyro P, 1.5 mgm. of acetaldehyde, 6 mgm. of Mg as $MgSO_4$. In Curves B and B + A the same solution without glucose. Solid curves: Curves G and B, no further additions; Curve O, saturated octyl alcohol; Curve O + P, saturated octyl alcohol + 0.25 cc. of 0.1 *M* $KH_2PO_4$; Curve O + 2P, saturated octyl alcohol + 0.25 cc. of 0.2 *M* $KH_2PO_4$. Dotted curves: Curve A, 0.25 cc. of 0.04 *M* arsenate; Curve A + O, 0.25 cc. of 0.04 *M* arsenate with saturated octyl alcohol; B + A, blank + arsenate. The arrow at 2 cc. shows the maximum amount of $CO_2$ which can form from the added glucose.

in Fig. 1 where a special inhibitor for ATP-ase is used, octyl alcohol, which like other higher members of the narcotic series inhibits the adsorbed ATP-ase of yeast, but not the same enzyme after it is dissolved and purified (6).

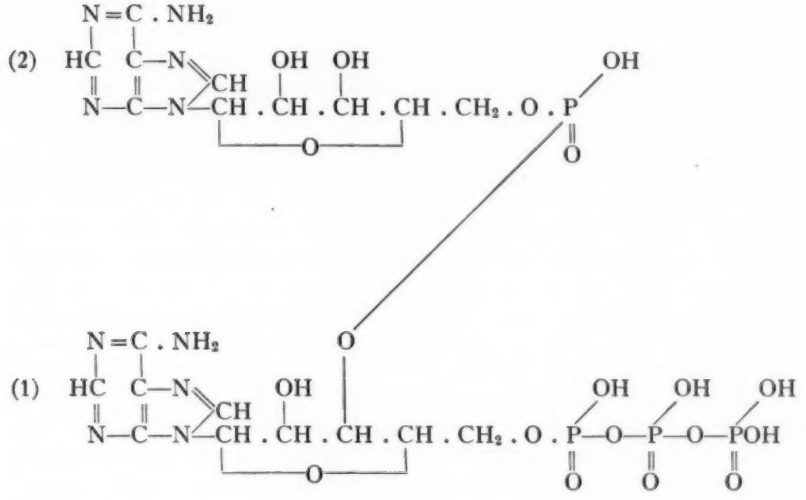
Fig. 1 shows that the quickly dried yeast containing undamaged ATP-ase ferments the sugar until exhaustion with nearly constant speed. This speed is the same in the presence of arsenate which abolishes the Harden–Young effect. If the ATP-ase is inhibited by octyl alcohol, then the two periods of the Harden–Young effect appear depending on the amount of phosphate and sugar present. The speed drops to a low level if either the phosphate (O and O + P) or the free sugar (O + 2P) are completely used up by forming an equivalent amount of HDP. In the presence of arsenate (A), (A +O), ATP-ase is unnecessary, and, therefore, does not influence t he speed, whether octyl alcohol is present or not.

Even this behavior of our new dry yeast cannot represent fully the enzymatic state of the living yeast. If we wonder why high energy phosphates should first be built up in the metabolic reactions and then degraded or wasted by the splitting of ATP-ase we should not forget that our quickly dried yeast corresponds at best to the living resting cell without any kind of growth and very little synthesis and no kind of work going on. But in the case that such activities like growth or synthesis would actually occur, then the high energy phosphate would be used for them. We can be pretty sure that the synthetic reactions needed for growth and also for osmotic and other work done by the cell are mediated by the energy rich phosphate bond. Even if we speculate why nature in the breakdown of carbohydrate has made the detour by way of phosphorylated intermediaries, we can find the easy answer that this is for the production of high energy phosphate compounds. In these the energy is stored either for transfer to other substances needed for synthesis or even for transfer into other forms of energy, as we know especially from the mechanical work of muscle.

For yeast itself we have recently more closely investigated the formation of metaphosphate which is probably mainly hexametaphosphate. We tried to separate the synthetic mechanism from the living cell and found some evidence although not conclusive, that it might go on in the presence of sugar and phosphate in a granular suspension obtained from yeast. This metaphosphate is undoubtedly a kind of high energy phosphate which is derived from ATP. In this form of hexametaphosphate the energy of fermentation would be stored in yeast in a similar way as it is stored in muscle in the form of phosphocreatine. As Wiame has found (16), if the yeast grows the polymetaphosphate disappears and is apparently used for the formation of nucleic acids. Here we see a new bridge between the glycolytic reactions and growth, a bridge formed by ATP and polymetaphosphate.

Besides ATP we have to consider still other cofactors for phosphorylation present in the living yeast cell. Twelve years ago Dr. Kiessling and I described a dinucleotide of the yeast which consisted of 1 mole ATP connected with 1 mole AMP by a ribose linkage (4). I show it in Scheme 6.

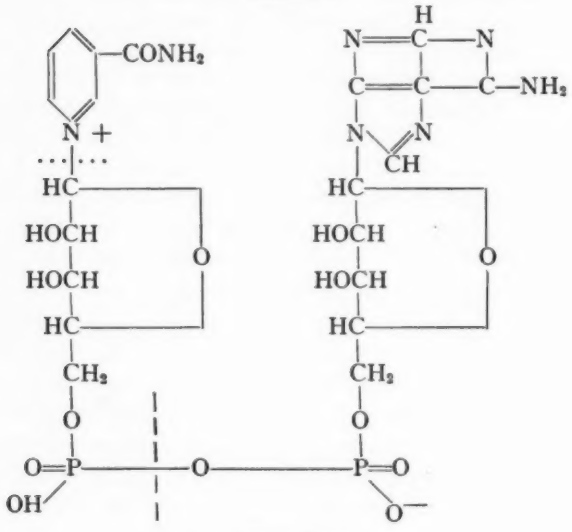
Scheme 6. Adenine dinucleotide of Meyerhof and Kiessling.



(Whether the oxygen bridge goes to the third or second carbon of the ribose unit is undetermined.) We were convinced that this dinucleotide is actually the form in which the adenylic system exists in the living yeast cell and that our usual mononucleotides, ATP, ADP, and so on, are artifacts. This difference did not seem to play a great role because the single transphosphorylation reactions which we studied at that time were equally well catalyzed by the dinucleotide or by ATP itself. But recently Dr. Ohlmeyer, who was formerly my assistant in Heidelberg, came to this country and prepared in Dr. Kornberg's laboratory in Bethesda an apparent dinucleotide from yeast which seems closely related to that of Kiessling if not identical (11). His cofactor increased the fermentation of yeast maceration juice very much more than ATP in the second period where the speed depends on the availability of inorganic phosphate. It seems that this nucleotide in the presence of a special enzyme can transfer and release phosphate faster than ATP itself. The question is not quite cleared up, but it reminds us that our schemes are mere skeletons and that some activating and inhibiting factors have already fallen away during the preparation of the various enzymes and coenzymes.

This is also true for the oxidation–reduction coenzyme. The DPN is not such an invariable molecule as it was formerly assumed when we were concerned only with the two steps of oxidation and reduction. Dr. Kornberg (5), as you know, has found in highly interesting work, that DPN can be split reversibly in the presence of inorganic pyrophosphate, to ATP and nicotinamide mononucleotide (NMN).

Scheme 7.



Cozymase

In Scheme 7, the formula of cozymase is given. The splitting (– – – –) described by Dr. Kornberg occurs between the two phosphate groups, while another enzyme, the DPN nucleosidase can split off the nicotinamide alone (· · · ·). I will not go into further details because I would like to discuss still another closely related system of glycolysis in animal tissue.

I will not come back to the older work on muscle where the system was first studied *in vivo* and *in vitro*. But in the last few years we studied these reactions in brain tissue and tumor tissue and also in the homogenates of these tissues. Although qualitatively these reactions are much the same as those which I discussed here in yeast, there is a great quantitative difference in regard to the activity of the enzymes. The ATP-ase of yeast is highly unstable and the hexokinase of yeast is relatively stable. In animal tissues the situation is reversed: the hexokinase is much more unstable than the ATP-ase. The latter one is apparently in excess.

We best compare the behavior of three tissue preparations in various stages of disintegration of the cells to learn something about the reactions in the living organ itself. The first is the tissue slice made according to Warburg (15). The second is the tissue homogenate made according to the general procedure of Potter and Elvehjem (12), and finally the third preparation is the tissue extract which is made from the homogenate by centrifuging and discarding the precipitate.

In the slices we have preserved the conditions of the living tissue except for hormones and regulators. They, therefore, need no addition of coenzymes or phosphate. When we express lactic acid formation by the notation introduced by Warburg of $Q_{La}^{N_2}$ for cubic mm. carbon dioxide driven out by lactic acid at 38° per mgm. dry weight per hour, then $Q$ is approximately 10 – 20 for 0.2% glucose and only 1 to 2 for 0.2% fructose.

I was especially intrigued by this difference of glucose and fructose because we knew that respiration of brain slices is the same with glucose and fructose. The experiments with homogenates and extracts finally gave a full explanation for this curious difference. When we make the homogenate with a modified Ringer solution and so thorough a homogenization that all cells are destroyed, then addition of cozymase, nicotinamide, and ATP becomes necessary for maintaining a continuous glycolysis. Moreover, much depends on the substrate, whether we use only glucose as does the living brain or a mixture of glucose and HDP. The latter mixture used by Utter and others is much more favorable for a high glycolysis because the phosphate acceptor, glucose, and the phosphate donor, HDP, balance each other (14). However, this blurs somewhat the clear picture of what would go on in the living cell. In the next figure, Fig. 2, you see how the glycolysis in such homogenates behaves (8).

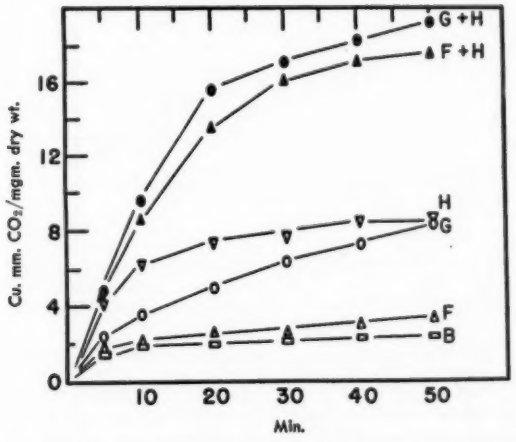



FIG. 2. Glycolysis in complete homogenate with glucose and fructose, with and without HDP addition; 0.3 cc. homogenate (15 mgm. dry weight) in 1.2 cc. total volume in each sample. ▽ H = HDP with 450 γ hexose (112 cu. mm. $CO_2$), which can give in maximo 7.5 cu. mm. per mgm. dry weight. ○ G = glucose (4 mgm.); △ F = fructose (4 mgm.); □ B = blank; ● G + H and ▲ F + H = mixtures of 4 mgm. glucose or fructose with 450 γ hexose of HDP.

Actually, the $Q$ values of glucose and fructose are roughly the same as are found in slices, showing the same difference. But this difference nearly disappears when one uses a mixture of sugar and HDP. Since a homogenate does not contain intact cells, this difference between the two sugars has nothing

to do with permeability. This can be demonstrated still more strikingly if one centrifuges the particles out and tests the extracts for glucose and fructose turnover.

In the following picture (Fig. 3A) we see not only again the rate for glucose and fructose in the homogenate but the startling effect which results from centrifuging away the particles. The clear enzyme extract shows a rate of glycolysis which is eight times as high as in the homogenate from which the extract was obtained. Moreover, there is now no more difference between glucose and fructose.

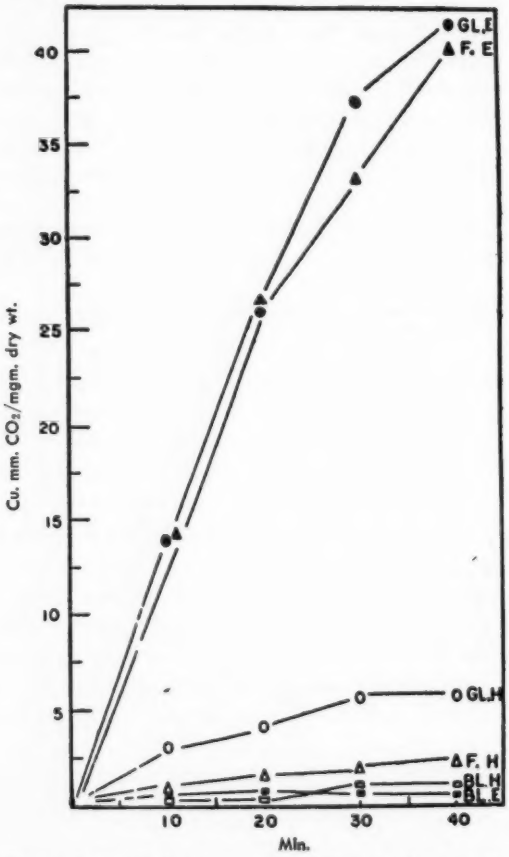



FIG. 3A. Glycolysis in complete homogenate (H) and, after centrifuging, in extract (E). Homogenate frozen and thawed (Gl. 119). Vol. 0.75 cc. containing 0.2 cc. homogenate or extract (1 : 3) with Ca-free Ringer, bicarbonate, and $MgSO_4$; nicotinamide to 0.6% final concentration; 0.05 cc. hexose diphosphate (20 γ P); two minutes before start 0.05 coz. (0.3 mgm. DPN), 0.05 cc. ATP (30 γ seven minutes P), 0.05 cc. isotonic bicarbonate tipped in. □ ■ Bl. E and H = blank of extract and homogenate; ● Gl. E and ○ Gl. H = 3 mgm. glucose in extract and in homogenate; ▲ F. E and △ F. H = 3 mgm. fructose in extract and in homogenate.

Fig. 3 B shows the same, but besides the homogenate I used an artificial mixture of washed brain particles and brain extract, a mixture which behaves like the homogenate (7). Former authors occasionally spoke of a glycolysis

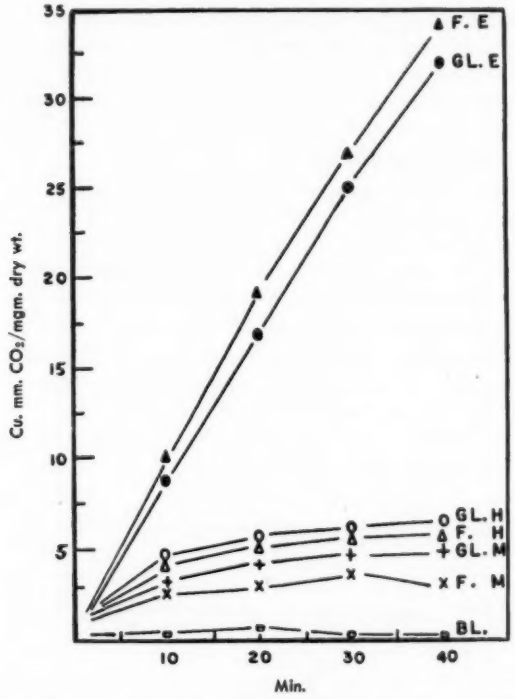



FIG. 3B. A similar experiment where besides 0.15 extract (F. E and Gl. E) also a mixture of 0.2 washed particles with 0.15 cc. extract is used (Gl. M and F. M). This mixture gives even lower values than the uncentrifuged homogenate (Gl. H and F. H).

inhibitor contained in the particulate matter of the brain. Such an inhibitor does not exist. The striking difference whether the particulate matter is removed or not, stems from the excess of ATP-ase which is firmly bound to the particles. This excess ATP-ase diminishes the ATP content to a very low level. If we centrifuge the particles out, we remove this excess and now phosphorylation and dephosphorylation are brought into step. This alone is the reason for the high glycolysis in the extract.

The same explanation holds also for the difference of glucose and fructose glycolysis which exists in slices and homogenates but which disappears in the enzyme extract.

Closer analysis reveals that this difference between the two sugars is caused by a different affinity to ATP in the presence of hexokinase. In the brain

only one single hexokinase exists, as is now the general consensus of various authors (Quastel (2), Slein and Cori (13), and myself), and not a separate fructokinase and glucokinase as the Coris have found in liver and muscle. But because the affinity of fructose for the enzyme is lower or to say the same, the dissociation constant of the enzyme substrate complex is much greater, a difference of reaction rate of glucose and fructose becomes apparent with low ATP as well as with low sugar concentration. For the homogenate we do not exactly know how low the ATP concentration becomes on account of the ATP-ase. But with the extracts we can do quantitative experiments. I show it here on the next figure, Fig. 4.

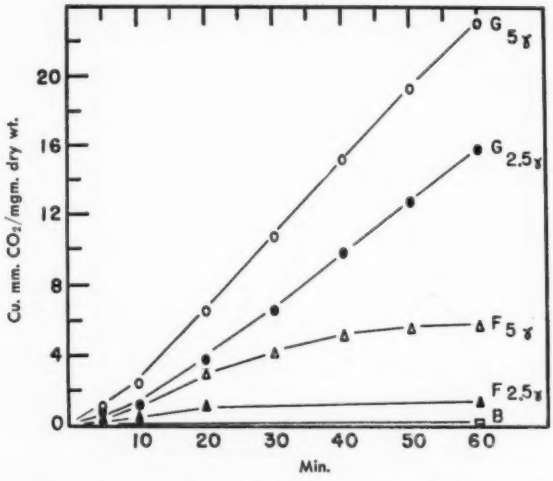



FIG. 4. Decrease of speed of glycolysis with decreased ATP concentration ($\gamma$ refers to the pyro P of ATP). Glycolysis of glucose and fructose with very low ATP concentrations. All samples contain 0.95 cc. total volume, 0.3 cc. extract (1 part tissue to 3.3 parts modified Ringer solution), $1 \times 10^{-2}$ *M* glutathione, $0.3 \times 10^{-2}$ *M* $NaHCO_3$ $5 \times 10^{-3}$ *M* phosphate, 0.3 mgm. DPN, HDP with 7 $\gamma$ P and 2 mgm. sugar.

B □ = blank; $G_{5\gamma}$ ○ —— ○ 1 mgm. glucose, 5$\gamma$ pyro P of ATP; $F_{5\gamma}$ △ —— △ 1 mgm. fructose, 5$\gamma$ pyro P of ATP; $G_{2.5\gamma}$ ● —— ● 1 mgm. glucose, 2.5$\gamma$ pyro P of ATP; $F_{2.5\gamma}$ ▲ —— ▲ 1 mgm. fructose, 2.5$\gamma$ pyro P of ATP.

If one lowers the amount of pyro P or ATP to 5 $\gamma$ P per 1 cc. or to $10^{-4}$ *M* instead of the normal concentration of $10^{-3}$ *M*, then the fructose reacts much more slowly, as you see here. If we halve the amount again, glucose itself reacts somewhat more slowly, but fructose practically not at all. Here we have re-established in the extract the conditions prevailing in slices and homogenates. We can do nearly the same in keeping the ATP concentration high and decreasing the sugar concentration much below 0.2%. I show you on Scheme 8, the results of such an experiment (9).

The ATP concentration was left high, $2 \times 10^{-3}$ *M*, but the sugar was reduced from 0.3% to 0.02% or from 22 to 1.7 $\mu$moles (in 1.3 cc.). With normal sugar concentration transphosphorylation is equal for both sugars

Scheme 8.

HEXOKINASE IN BRAIN, AMOUNTS IN μMOLES PER 1.3 CC. 2.2 ATP, 12 MIN. 38°

| Added sugar | | P-transfer (7 min. P decrease) | Sugar decrease |
|---|---|---|---|
| Glucose | 22.2 | 1.62 | — |
| Fructose | 22.2 | 1.88 | — |
| Glucose | 1.7 | 1.78 | 1.30 |
| Fructose | 1.7 | 0.85 | 0.66 |

but it is one-half as much for fructose in the low range of sugar. The same can be found by measuring the sugar consumption directly (last column). Wiebelhaus and Lardy (17) calculated from their own later experiments which were similar to those here described that the Michaelis–Menten constant for the dissociation of the enzyme–fructose complex would be $10^{-3}$. This value also would come out from my own experiments as given in Scheme 8. However, the application of this constant to a bimolecular reaction is questionable, the more so as the dissociation is influenced by the ATP concentration more strongly than by the sugar concentration. We find the same difference for the low concentrations of sugars also by measuring the glycolytic rate as is shown in Fig. 5.

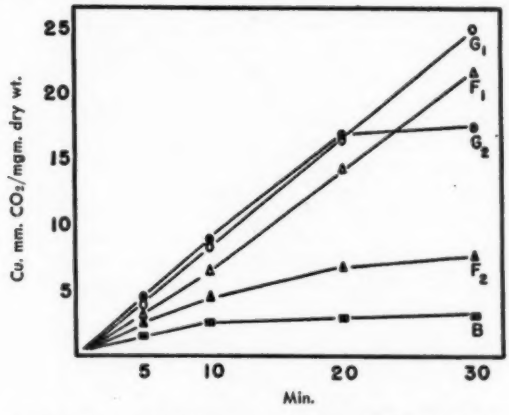



FIG. 5. Glycolysis in centrifuged extract; 0.3 cc. extract, 1 : 4.6 in 1.5 cc. total volume containing sugar as well as a trace of HDP (17 γ P) as primer; 0.1 cc. cozymase (0.3 mgm. DPN), 0.1 cc. ATP (35 γ pyro P), 0.05 cc. isotonic sodium bicarbonate is tipped in from the side arm at the start. B = blank; $G_1$ and $F_1$ = 3 mgm. glucose and 3 mgm. fructose, respectively; $G_2$ and $F_2$ = 0.6 mgm. glucose and 0.6 mgm. fructose, respectively.

If you apply these findings to the living cell, it seems that the more important factor is the ATP concentration. The acting concentration of ATP is apparently much lower than that found by analysis. Either the

organization of the cell hinders the ATP from being distributed equally or it is in a large part bound in an inactive complex. The difference between the glucose and fructose glycolysis in slices becomes immediately understandable if we assume that only 5 to 10% of the ATP is available to hexokinase. Because of this low affinity of fructose, it is not able to replace glucose. Indeed, as Cori and Slein have found, phosphorylation of fructose is nearly completely inhibited by the presence of glucose in the same concentration. This may explain why fructose cannot relieve the symptoms of hypoglycemia in brain. But mannose can do it. Indeed, the affinity of mannose is nearly the same as that of glucose in brain extracts.

I have investigated in the same manner the glycolytic mechanism in malignant tumor (10). Although some points are different, the general pattern is roughly the same as in brain. In this case a new device is used for inhibiting the excess of ATP-ase; that is, an addition of octyl alcohol which as a high member of the narcotic alcoholic series, inhibits the tumor ATP-ase in a similar way as the adsorbed ATP-ase of yeast. It must be said, however, that the ATP-ase of tumor is in solution or perhaps in a fine suspension which cannot be centrifuged out by ordinary centrifugation.

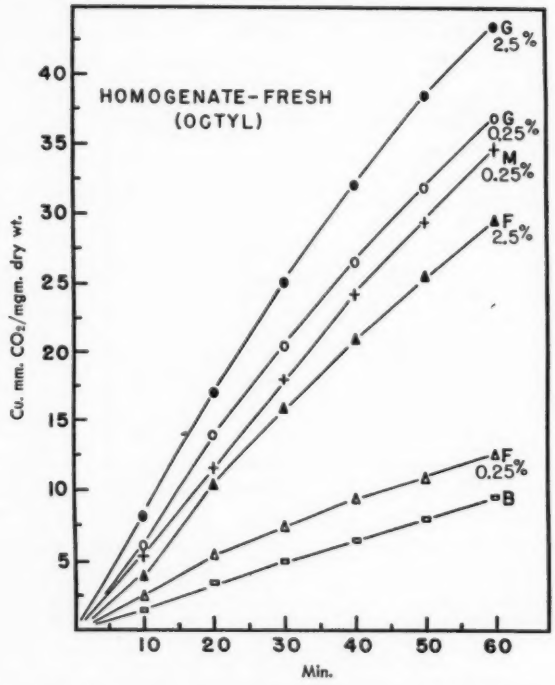



FIG. 6. Glycolysis of various sugars in the homogenate of fresh tumor in the presence of octyl alcohol. B = blank; G = glucose; M = mannose; F = fructose. The curve for 2.5% mannose, not drawn, nearly coincides with that of 2.5% glucose.

As Fig. 6 shows, 0.25% glucose and mannose give the same glycolytic rates; 2.5% glucose very little more. However, fructose behaves differently; 0.25% shows very little more glycolysis than the blank while 2.5% approaches the speed of a 10 times lower concentration of glucose. We may state furthermore that roughly the concentration of the glycolytic enzymes seems greater in tumor than in brain. However, this may not be enough to explain the higher glycolysis of malignant tumor tissue which is about three times as high as it is in brain tissue. Actually, there is so much excess enzyme present in these tissues that glycolysis could still be much higher but for the regulating influences of various factors. One such regulating factor I have demonstrated here, the acting ATP concentration. In the presence of the phosphorylating and dephosphorylating enzymes it determines the speed of glycolysis in general as well as the relative speed of the various sugars. This shows at the same time how far we can apply the results of enzyme studies to the living cells; but it does not, of course, include the influence of hormones and other regulators nor the specific organization of the living structures in modifying the speed of the basic metabolic reactions.

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